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EXPLORING THE COFACTOR-BINDING AND BIOCATALYTIC PROPERTIES OF FLAVIN-CONTAINING ENZYMES

Małgorzata M. Kopacz

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CHAPTER 1

GENERAL INTRODUCTION

ENZYMES AS NATURE'S CATALYSTS

Each living organism relies on very specific catalysts, known as enzymes. Enzymes are proteins whose structure determines the shape of an active site and therefore the accepted substrates. Because of their regio- and stereospecificity, enzymes are extremely powerful catalysts. Furthermore, they can perform almost any type of chemical reaction, usually under mild conditions. Increasing demands to reduce pollution make biocatalysts an interesting and green alternative to current industrial applications [1–3]. For example, proteases and lipases are widely used in laundry detergents. Amongst other applications glucose oxidase is utilized for the determination of glucose concentration and is one of the most widely applied analytical enzymes [4]. In recent years research focussing on the discovery and engineering of enzymes has intensified to assist the development of new biorefinery processes, for example enzymes for the production of biodegradable polymers or biofuels.

The exquisite specificity of enzymes has probably evolved from the delicate physiological role they play. This property makes them interesting tools in synthetic chemistry. However, the limited number of suitable substrates and enzyme-catalysed reactions has so far been their major drawback. Two strategies are employed to broaden the capability of already known enzymes. The first method is to search for new activities in various environments and to screen available enzyme collections (databases), the latter having gained more popularity in recent years. The other possibility is the introduction of the required properties into already existing enzymes, such as substrate scope and specificity, activity, stability and the expression level. Depending on the extent of knowledge about the target enzyme, two main approaches are generally available. If the understanding of the sequence-structure-function relationship is limited, the random approach is the most suitable [5]. The drawback is that it creates a large number of variants and therefore requires a high-throughput screening method. The opposite, the rational approach, depends on the structural information and typically involves relatively small libraries. Therefore, extensive screening is not necessary in the latter approach, but it still often suffers from low predictability and reliability. The efficacy can be improved by employing more powerful computational design methods [6]. This is not only the result of an increase in computational power, but also from improvement in scoring and search functions in modelling [7,8].

OXIDOREDUCTASES

Enzymes are divided in six classes, depending on the type of reaction they catalyse. Enzymes that transport electrons between molecules are called oxidoreductases, or redox enzymes and they belong to the first class of enzymes (E.C. 1.x.x.x). While a number of cofactor-independent oxidoreductases is known, most of them make use of (in)organic cofactors for the reaction they catalyse [9]. This is because the chemical functionality of amino acids is limited, but the use of cofactors can broaden the scope of enzyme-catalysed reactions [9]. The most well-known cofactors used by redox enzymes are flavins, metal ions, hemes and nicotinamides. They are discussed in more detail in Chapter 2.

Reactions catalysed by oxidoreductases are very diverse and include the oxidation of amines, alcohols and aldehydes, the epoxidation of alkenes, Baeyer-Villiger oxidations, the hydroxylation of aromatic and aliphatic compounds and others. Based on the reaction they catalyse and their cofactor dependency they are usually divided into oxidases, oxygenases, dehydrogenases, peroxidases and peroxygenases. While dehydrogenases typically depend on a redox coenzyme (e.g. NAD(P)H or $NAD(P)^+$) to donate or accept electrons, oxidases, peroxidases and peroxygenases use molecular oxygen or peroxides as an electron acceptor. Since oxidases and monooxygenases will be the subject of this thesis, they are discussed in more detail below.

Oxidases catalyse two- or four-electron oxidations of substrates such as amines and alcohols and they are able to perform oxidative ring closure, oxidative decarboxylation and hydroxylation reactions [10]. The use of molecular oxygen as an electron acceptor is an energetically unfavourable strategy, because transfer of energy-rich electrons to dioxygen results in the formation of H_2O_2 or H_2O. Instead, the transfer of electrons to for instance $NAD(P)^+$ would produce a reduced coenzyme that can subsequently be used in other anabolic pathways. This is probably why oxidases are rather rare enzymes compared to dehydrogenases and they are typically involved in catabolic routes. Though, this is also the reason why they are well-suited for biotechnological applications because the use or regeneration of expensive nicotinamide coenzymes is not needed. Oxidases use flavin, copper or multi-redox centres as cofactor.

In contrast to oxidases that use oxygen as an electron acceptor, oxygenases insert one (monooxygenases) or two (dioxygenases) oxygen atoms into the

substrate. For this purpose oxygen has to be activated and this is usually achieved with electrons from the substrate itself or from an external electron donor such as NAD(P)H. Oxygenases are present in almost all kinds of living cells and are typically involved in detoxification, degradation pathways or the synthesis of secondary metabolites [11]. The specific oxyfunctionalisation reactions feasible with oxygenases are very interesting in industrial chemical synthesis [12].

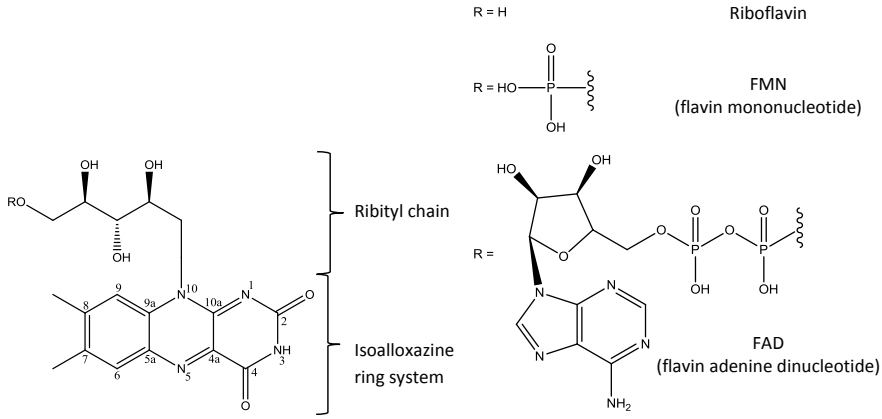

Figure 1.1. The structure of naturally occurring flavin cofactors.

FLAVOPROTEINS

Flavin cofactors

One type of cofactor commonly used by oxidoreductases is the flavin cofactor, although this metal-free cofactor can also be found in enzymes catalysing non-redox reactions. Discovered at the end of the XIX century and chemically characterized in the 1930's, flavins are able to catalyse both one- and two-electron transfer processes. Their name originates from the Latin word "flavus", which means "yellow", and also denotes the colour of flavoproteins. The two types of flavin cofactors most often found in nature, namely flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Fig. 1.1), are both synthesized from riboflavin (vitamin B2). They are composed of the isoalloxazine ring moiety, which is responsible for the yellow colour in the oxidized state. On the other hand, two-electron reduced flavins hardly absorb in the visible region and therefore are

colourless. The oxidized forms of FAD and FMN can also accept a single electron at a time forming a half-reduced flavin called semiquinone which has distinctive spectral properties. In addition to exhibiting absorbance, the oxidized flavins are characterized by fluorescence emission spectra with a maximum of emission at approximately 520 nm. The fully reduced or semiquinone flavin is nonfluorescent in aqueous medium at ambient temperatures.

The solubility of riboflavin in water is relatively low ($<10^{-4}$ M) while the solubility of FMN and FAD is high (approximately 0.1 M), caused by the latter two having negatively charged phosphorylated side chains. The chemical properties of flavins are determined by the three fused ring systems, namely xylene, pyrazine and pyrimidine. The xylene ring moiety is relatively rich in electrons compared to the electron deficient pyrimidine moiety. Most of the flavin chemistry occurs on the bridging ring, the pyrazine moiety. The electronegativity of the 4a-position originates from the four nitrogen atoms and two carbonyl groups in the pyrazine and pyrimidine rings. The pyrimidine ring moiety is hydrophilic and can interact with protein residues as a hydrogen donor or acceptor, influencing the properties of the pyrazine ring in this way. The xylene moiety is hydrophobic and the typical interactions found for this part of the isoalloxazine ring are stacking interactions with side chains of protein aromatic amino acids. It is interesting to note that the protons on the 8-methyl position are much more labile to exchange than expected, when compared to the 7-methyl group protons. This originates from a very strong inductive effect of the N5 position [13]. The midpoint redox potential of the flavin is approximately -210 mV against a standard hydrogen electrode, but is slightly dependant on the nature of the substitution at the N10 position and strongly dependant on the substitutions and modifications in the pyrimidine ring as well as the benzene moiety at positions 8 and 6. Furthermore, the midpoint redox potential can be largely modified by the protein environment (see Chapter 4).

The most useful information about flavin-protein interactions can be obtained from the structural data, but also other sources are available, for instance the influence of substituting the native flavin with its analogue in the protein. In order to do so, the protein has to be (partially) unfolded in a non-destructive way and incubated with a flavin analogue which should result in a functional reconstituted holo protein. The situation is more challenging in the case of flavoproteins that bind the cofactor covalently. Here, the strategy depends on the stability of the apo form of the protein. If the stability is not an issue, the protein can be expressed

in a riboflavin-auxotrophic strain and purified in its apo form and subsequently reconstituted with the flavin. In the second case, a riboflavin-auxotrophic strain has to be supplied with the riboflavin analogue in order to express the studied flavoprotein as a holo protein. The approach of flavin substitution has proven to be of great value, because flavin analogues can be used as probes for (covalent) flavin-binding and protein environment analysis, as well as for the investigation of flavoenzyme activity and mechanism (see reviews: [14–18]). Additionally, the combination of artificial flavins with different protein scaffolds can result in the formation of enzymes with new catalytic activity [19,20] (and Chapter 2).

Flavin-containing oxidases

One of the cofactors used by oxidases is a flavin and therefore these enzymes are referred to as flavin-containing or flavoprotein oxidases. Flavoprotein oxidases utilize FAD as a prosthetic group more often than FMN [21] and usually it is the only cofactor. A comparison of currently available sequences and structures indicates that flavoprotein oxidases belong to several structurally distinct families [22]. Here, only two of them will be discussed, namely the vanillyl-alcohol oxidase (VAO) and the amine oxidase (AO) families.

The VAO family has been named after the fungal flavoprotein oxidase, VAO [23,24]. It was the first flavoprotein with a covalently bound flavin for which the crystal structure was elucidated [25]. Covalent flavin binding is relatively abundant in this family, which also includes all recently discovered flavoproteins with two covalent bonds to the flavin [26]. VAO-type oxidases are catalytically diverse, because they can perform not only alcohol oxidations, which lead to aldehydes and ketones [27–29], but also amine oxidations, hydroxylations of aromatic compounds, decarboxylations, and ether bond cleavage [30,31]. All these reactions typically exhibit excellent chemo-, regio- and enantioselectivity [32]. These examples suggest that the VAO-type of protein fold is capable of performing a great number of diverse oxidation reactions. A large number of new VAO-type enzymes were found and studied recently [24,33], including reticuline oxidase (see below). The VAO-family also contains the first discovered flavoprotein with a covalent linkage, namely 6-hydroxy-D-nicotine oxidase (6HDNO) [34,35], which is the subject of Chapter 4. For a long time, 6HDNO was considered the prototype for a covalent flavoprotein.

Prototype oxidases for the AO family are microbial amino acid oxidases and eukaryotic monoamine oxidases. In addition to the oxidation of primary and secondary amines, a few members of the family have been found to oxidize alcohols and thioethers, but usually an individual enzyme is restricted to only one type of reaction. Human monoamine oxidases A and B (MAO A and MAO B) have been the subject of intense studies for a long time, because they catalyse oxidation of endogenous amine neurotransmitters like serotonin and dopamine, as well as many amines present in food and drugs. Although they have the same overall structure, MAO A has a more open active site [36,37] and for this reason a small difference in substrate scope is observed. A number of MAO inhibitors have been developed for clinical use for treatment of depression and neurodegenerative diseases like Alzheimer and Parkinson. However, their many side effects have shifted the interest into the design of inhibitors specifically targeting each form of MAO [36,38]. In this thesis, a bacterial member of the MAO family, putrescine oxidase, will be discussed.

Flavoprotein oxidases have been successfully applied in the biotechnological industry, mostly because of their interesting catalytic properties owing to a wide variety of oxidative reactions with excellent chemo-, regio- and/or enantioselectivity. Furthermore, they use molecular oxygen as a mild oxidant. In addition to the previously mentioned glucose oxidase, several other flavin-containing oxidases have been applied in a variety of processes. D-amino acid oxidase has been employed in the synthesis of antibiotics [39] and fungal monoamine oxidase in the production of enantiopure fine chemicals [32]. Interestingly, flavoprotein oxidases have been found to not only catalyse relatively simple oxidations, such as the conversion of alcohols to aldehydes and ketones, but also more complex reactions. For example, the plant enzyme reticuline oxidase (also known as berberine bridge enzyme) takes part in an oxidative C-C bond formation in (*S*)-reticuline, creating the intramolecular bond between a methyl group and an aromatic carbon, resulting in (*S*)-scoulerine (Fig. 1.2) [40,41].

Flavoprotein monooxygenases

Another group of interesting flavin-containing enzymes for biocatalytic purposes are flavoprotein monooxygenases. They can perform specific oxygenations, which

are difficult to obtain with chemical routes. Flavoprotein monooxygenases can be encoded by a single gene or can have multiple components. Based on sequence and structural data they can be divided into 6 distinct classes, where classes A and B are the most interesting as biocatalysts, because they are single component enzymes that contain a tightly bound flavin cofactor [42]. Within class B four subclasses are found: (1) Baeyer-Villiger monooxygenases (BVMOs) performing Baeyer-Villiger oxidations and heteroatom-containing compounds oxygenations [43]; (2) type I [44–47] and recently discovered (3) type II [48,49] flavin-containing monooxygenases (FMOs) oxidizing heteroatom-containing compounds and (4) N-hydroxylating monooxygenases (NMOs) converting long-chain primary amines by N-hydroxylation [50]. Due to the eukaryotic origin and membrane association, FMOs were initially not considered for their use as biocatalysts. This has changed recently with the discovery and soluble expression of microbial FMOs, one of them being described in this thesis.

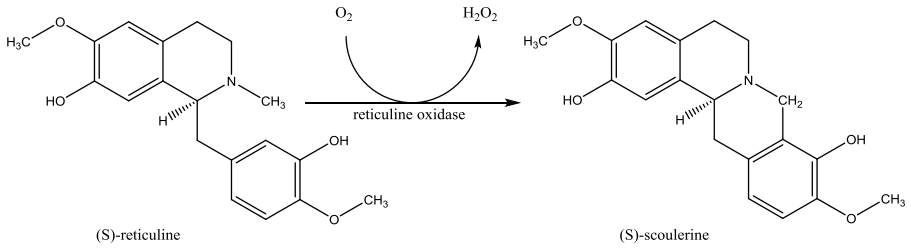

Figure 1.2. Oxidative C-C bond formation catalysed by reticuline oxidase.

COVALENT FLAVIN BINDING

Many cofactor-containing proteins bind their cofactor in a non-covalent manner. However, some specific cofactors, like lipoic acid and biotin, are always bound covalently to the polypeptide amino acids. Heme and flavin cofactors are the only cofactors that can be bound either covalently or noncovalently, depending on the enzyme. Although most flavoproteins contain a noncovalently bound flavin, it has been estimated that approximately 10% of them bind the cofactor covalently [26]. Furthermore, several different types have been discovered so far: 8α-N^3-histidyl-FAD/FMN, 8α-N^1-histidyl-FAD/FMN, 8α-*S*-cysteinyl-FAD/FMN,

8α-*O*-tyrosyl-FAD, 8α-*O*-aspartyl-FAD, 6-*S*-cysteinyl-FMN, 8α-N^1-histidyl-6-*S*-cysteinyl-FAD/FMN and threonyl-FMN (Fig. 1.3).

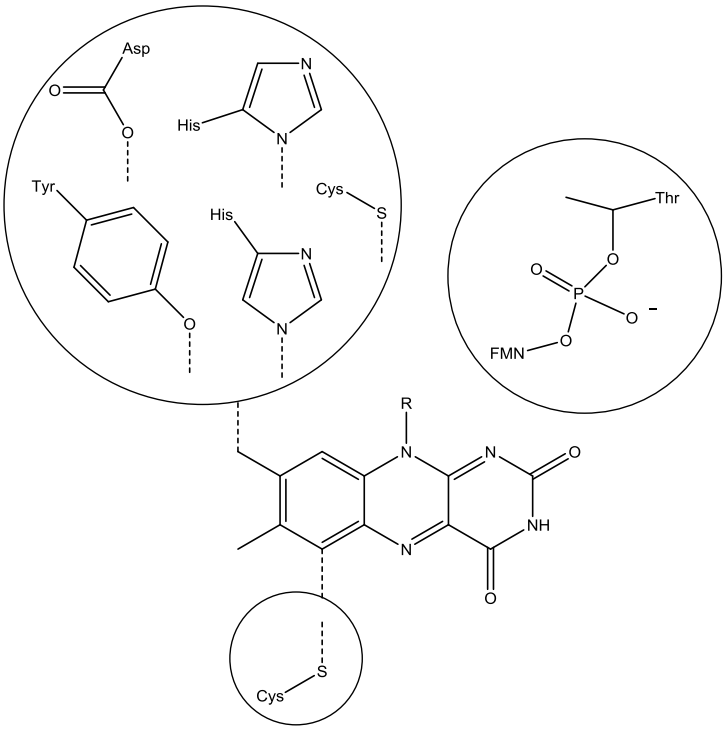

Figure 1.3. Possible modes of covalent flavinylation.

The first experimental data suggesting the existence of covalent flavoproteins appeared in the 1950s and the evidence was obtained from the work on succinate dehydrogenase which contains an 8α-N^3-histidyl-FAD cofactor [51,52]. The histidyl FAD type of linkage is the most common and can be found for example in VAO and alditol oxidase [28,53]. The most extensively studied flavoproteins with a cysteinyl linkage are monoamine oxidase, monomeric sarcosine oxidase (MSOX) and trimethylamine dehydrogenase (TMADH) [54–56]. The so-called bicovalent flavoproteins constitute a relatively new group of enzymes in which the bond between FAD and the polypeptide chain involves 8α-His and 6-*S*-Cys linkages. These enzymes are very interesting for biocatalytic applications, because they can catalyse bulky substrates, such as oligosaccharides, alkaloids and antibiotics

[29,41,57,58]. The reason for that is because the two covalent bonds between the flavin and the protein can properly position the flavin in a very open active site towards the substrate.

The covalent flavin binding seems to have more than one function and most information comes from site-directed mutants analysis. Covalent flavinylation increases the redox potential of the flavin and it has been shown that the flavin-histidyl linkage is directly responsible for this phenomenon [26]. The increased redox potential boosts the oxidative power of the enzyme and for this reason most of the (bi)covalent flavoenzymes are oxidases. Furthermore, covalent flavinylation might be necessary for some proteins to enhance their stability or to prevent the flavin dissociating from the binding site. In the case of trimethylamine dehydrogenase, the 6-*S*-cysteinyl-FMN precludes formation of 6-hydroxy-FMN, which leads to the enzyme inactivation [59].

In contrast to many other covalently linked cofactors which require the assistance of proteins to form a covalent cofactor-protein bond, in most cases the covalent flavinylation is catalysed by the flavoprotein itself. The proposed mechanism of this process was derived from studies on several monocovalent flavoproteins, like *p*-cresol methylhydroxylase (PCMH), 6HDNO, MSOX and TMADH [56,60–62]. For the 8α position, the first step of the proposed mechanism is the deprotonation of the C8 methyl group which leads to methide iminoquinone flavin formation (Fig. 1.4) [60,62–65]. The intermediate is expected to be stabilized at the N1-C2=O2 locus by a positively charged amino acid. This is followed by an attack on the 8α position from the residue that will form the covalent bond. In the last step the reduced flavin is reoxidized by molecular oxygen or another electron acceptor. The covalent flavinylation at the C6 position could proceed *via* a similar mechanism or by a direct attack of the cysteinyl group on the FMN. In the latter case formation of the iminoquinone methide intermediate would be omitted [56]. The bicovalent flavinylation has been studied to a lesser extent. The experiments on site-directed single mutants abolishing one of the covalent bonds have shown that the two bonds can be formed independently from each other [66,67]. Therefore both previously mentioned mechanisms could be valid for bicovalent flavoproteins, but it is unknown in which order these steps would take place and if any other processes are involved.

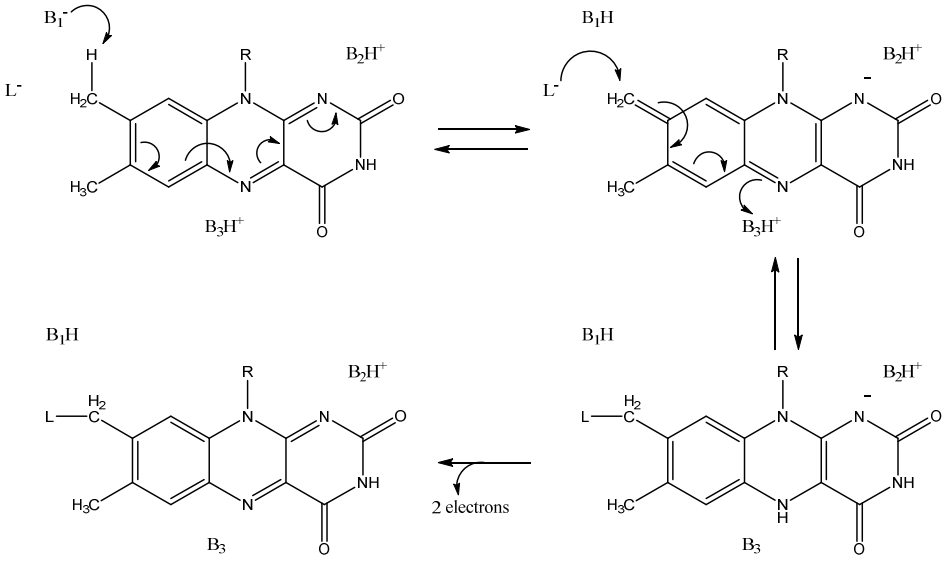

Figure 1.4. Proposed mechanism for covalent flavinylation at the 8α position in the isoalloxazine ring. B1-B3 represent active site bases potentially involved in covalent flavinylation and L stands for the ligand amino acid that covalently binds to the flavin and it can be histidine, tyrosine, cysteine or aspartate.

KINETIC MECHANISM OF FLAVOPROTEIN OXIDASES

Flavoprotein oxidases abstract two electrons and two protons from the substrate and these are further transferred on to a second substrate, which is molecular oxygen. Therefore, this catalytic mechanism can be divided into two half-reactions, namely a reductive and an oxidative half-reaction [68]. In the reductive half-reaction the flavin cofactor is reduced by a bound substrate, which is concomitantly oxidized to the product. The exact mechanism by which the two electrons and two protons are transferred to the flavin is still a matter of a debate for some flavoprotein oxidases. In the case of flavin-containing amine oxidases, three types of mechanism gained popular support: the concerted polar nucleophilic mechanism [69–71], the direct hydride transfer mechanism [72,73] and the single electron transfer mechanism [72,74]. However, the hydride transfer mechanism (Fig. 1.5) is the most consistent with the structural, mechanistic and computational studies on amine oxidases [72,75–80].

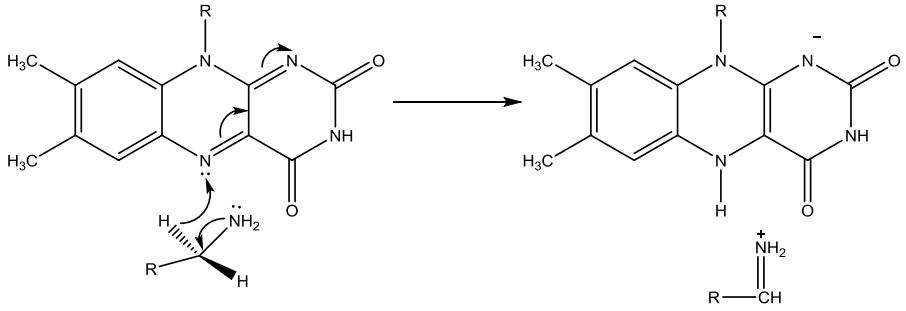

Figure 1.5. Hydride transfer mechanism proposed for the reductive half-reaction of flavin-dependent amine oxidases.

In order to close the catalytic cycle, the reduced flavin is reoxidized by molecular oxygen in the oxidative half-reaction, which results in the formation of hydrogen peroxide. The free reduced flavin reacts rather poorly with oxygen (2.5×10^2 $M^{-1}s^{-1}$ [81]), while in flavoprotein oxidases this reaction can be increased by a 10,000-fold or more, as for example in glucose oxidase (1.6×10^6 $M^{-1}s^{-1}$ [82]). This low reactivity of free reduced flavin can be explained by the law of spin conservation, because it involves the reaction of a singlet-reduced flavin with dioxygen in the triplet state. It has been proposed that in order to overcome this restriction, two one-electron transfers take place from the reduced flavin to oxygen instead of a single two-electron transfer [83,84]. The ability of a flavoprotein oxidase to use molecular oxygen is believed to be determined by the thermodynamic driving force formed by the flavin and O_2/O_2^- couple redox potentials, diffusion of oxygen into the active site, flavin stereochemistry and ligand binding [84,85].

Oxidases catalyse reactions starting with two substrates and resulting in two products. Therefore, they can follow several different kinetic mechanisms, for example a ping-pong or a ternary complex mechanism (Fig. 1.6). Flavoprotein oxidases can follow different types of mechanism and sometimes one enzyme can employ different mechanisms for different substrates. The kinetic mechanisms can be studied by analysing the reductive and oxidative half-reactions and this is usually done by using stopped-flow techniques. Flavoprotein oxidases are very convenient enzymes for this method, because their spectral properties depend on the redox state. In this thesis a kinetic analysis of putrescine oxidase is presented.

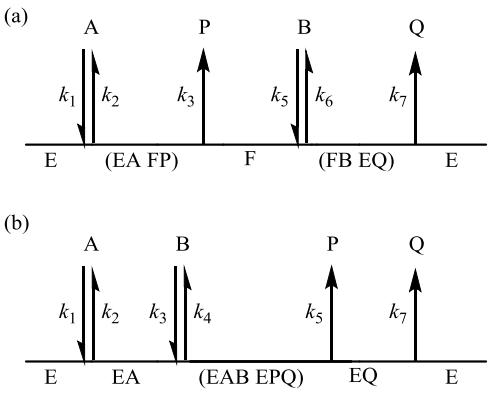

Figure 1.6. Typical kinetic mechanisms with two substrates (adapted from [86]): (a) ping-pong mechanism and (b) ternary complex mechanism. E is an enzyme, F is a modified enzyme, A and B represent substrates, P and Q represent products and intermediate enzyme complexes are in parentheses.

OUTLINE AND AIM OF THE THESIS

The aim of this thesis was the engineering of flavoprotein oxidases through cofactor binding and active site redesign. Furthermore, the biocatalytic scope of a bacterial flavin-containing monooxygenase was explored. Parallel to this, we aimed to prepare an isotopically labelled flavin to enable covalent flavinylation investigations.

The majority of this thesis focuses on flavin cofactor binding study and redesign. **Chapter 2** reviews the current knowledge about (in)organic cofactors, with the focus on redox cofactors, in particular nicotinamide, heme and flavin cofactors. Furthermore, novel approaches to cofactor and cofactor-containing enzymes redesign are presented.

Chapter 3 describes the first structural analysis of a bacterial amine oxidase, namely putrescine oxidase from *Rhodococcus erythropolis*. Furthermore, the crystal structure is a basis for the investigation of enzyme affinity towards the flavin, as well as for the design of artificial covalent cofactor binding.

In **Chapter 4**, a second covalent flavin-protein bond is introduced to a monocovalent flavoprotein, turning 6-hydroxy-D-nicotine oxidase into a bicovalent flavoprotein. This work indicates a histidine is necessary for the formation of a second bond and presents the new properties of the modified enzyme.

Chapter 5 is dedicated to the synthesis of isotopically labelled flavins and the conditions for specific flavin deuterations are presented.

In **Chapter 6**, a conserved amino acid in the active site of putrescine oxidase is mutated. With a single residue substitution, the narrow substrate specificity of putrescine oxidase is broadened towards diamines with a longer carbon chain.

Chapter 7 deals with a detailed pre-steady state kinetic study on putrescine oxidase. The enzyme reoxidation rate is increased by ligand binding in the active site. Mechanistic calculations suggest that putrescine oxidase follows a bifurcated mechanism and the ratio between the two contributing mechanisms depends on the oxygen concentration.

Chapter 8 describes the biocatalytic study on a flavin-containing monooxygenase from *Methylophaga* sp. (mFMO). The applicability of the enzyme for the preparation of colourful indigo derivatives is analysed.

REFERENCES

[1] H.E. Schoemaker, D. Mink, M.G. Wubbolts, Dispelling the myths – biocatalysis in industrial synthesis., Science. 299 (2003) 1694–1697.

[2] R.A. Sheldon, E factors, green chemistry and catalysis: an odyssey., Chem. Commun. (Camb). (2008) 3352–3365.

[3] R. Wohlgemuth, The locks and keys to industrial biotechnology., N. Biotechnol. 25 (2009) 204–213.

[4] S.B. Bankar, M. V. Bule, R.S. Singhal, L. Ananthanarayan, Glucose oxidase – An overview., Biotechnol. Adv. 27 (2009) 489–501.

[5] H. Leemhuis, R.M. Kelly, L. Dijkhuizen, Directed evolution of enzymes: Library screening strategies., IUBMB Life. 61 (2009) 222–228.

[6] J. Damborsky, J. Brezovsky, Computational tools for designing and engineering biocatalysts., Curr. Opin. Chem. Biol. 13 (2009) 26–34.

[7] D. Röthlisberger, O. Khersonsky, A.M. Wollacott, L. Jiang, J. DeChancie, J. Betker, et al., Kemp elimination catalysts by computational enzyme design., Nature. 453 (2008) 190–195.

[8] L. Jiang, E.A. Althoff, F.R. Clemente, L. Doyle, D. Röthlisberger, A. Zanghellini, et al., *De novo* computational design of retro-aldol enzymes., Science. 319 (2008) 1387–1391.

[9] J.D. Fischer, G.L. Holliday, S.A. Rahman, J.M. Thornton, The structures and physicochemical properties of organic cofactors in biocatalysis., J. Mol. Biol. 403 (2010) 803–824.

[10] L.M. Blank, B.E. Ebert, K. Buehler, B. Buhler, Redox biocatalysis and metabolism: molecular mechanisms and metabolic network analysis, Antioxid. Redox Signal. 13 (2010) 349–394.

[11] J.B. van Beilen, W.A. Duetz, A. Schmid, B. Witholt, Practical issues in the application of oxygenases., Trends Biotechnol. 21 (2003) 170–177.

[12] J.M. Bollinger, J.B. Broderick, Frontiers in enzymatic C-H-bond activation., Curr. Opin. Chem. Biol. 13 (2009) 51–7.

[13] S. Ghisla, D.E. Edmondson, Flavin Coenzymes, Encycl. Life Sci. (2009) 1–9.

[14] S. Ghisla, V. Massey, New flavins for old: artificial flavins as active site probes of flavoproteins., Biochem. J. 239 (1986) 1–12.

[15] C. Walsh, Flavin Coenzymes: At the Crossroads of Biological Redox Chemistry, Acc. Chem. Res. 13 (1980) 148–155.

[16] V. Massey, The chemical and biological versatility of riboflavin, Biochem. Soc. Trans. 28 (2000) 283–296.

[17] M.H. Hefti, J. Vervoort, W.J.H. Van Berkel, Deflavination and reconstitution of flavoproteins, Eur. J. Biochem. 270 (2003) 4227–4242.

[18] A.H. Merrill, J.D. Lambeth, D.E. Edmondson, D.B. McCormick, Formation and mode of action of flavoproteins, Annu. Rev. Nutr. 1 (1981) 281-317

[19] H.L. Levine, Y. Nakagawa, E.T. Kaiser, Flavopapain: Synthesis and properties of semi-synthetic enzymes, Biochem. Biophys. Res. Commun. 76 (1977) 64–70.

[20] G. de Gonzalo, C. Smit, J. Jin, A.J. Minnaard, M.W. Fraaije, D.A. Links, Turning a riboflavin-binding protein into a self-sufficient monooxygenase by cofactor redesign, Chem. Commun. (Camb). 47 (2011) 11050–11052.

[21] P. Macheroux, B. Kappes, S.E. Ealick, Flavogenomics – a genomic and structural view of flavin-dependent proteins, FEBS J. 278 (2011) 2625–2634.

[22] W.P. Dijkman, G. de Gonzalo, A. Mattevi, M.W. Fraaije, Flavoprotein oxidases: classification and applications, Appl. Microbiol. Biotechnol. 97 (2013) 5177–5188.

[23] M.W. Fraaije, W.J.H. van Berkel, J.A.E. Benen, J. Visser, A. Mattevi, A novel oxidoreductase family sharing a conserved FAD-binding domain, Trends Biochem. Sci. 23 (1998) 206–207.

[24] N.G.H. Leferink, D.P.H.M. Heuts, M.W. Fraaije, W.J.H. van Berkel, The growing VAO flavoprotein family, Arch. Biochem. Biophys. 474 (2008) 292–301.

[25] A. Mattevi, M.W. Fraaije, A. Coda, W.J. van Berkel, Crystallization and preliminary X-ray analysis of the flavoenzyme vanillyl-alcohol oxidase from *Penicillium simplicissimum*., Proteins. 27 (1997) 601–603.

[26] D.P. Heuts, N.S. Scrutton, W.S. McIntire, M.W. Fraaije, What's in a covalent bond? On the role and formation of covalently bound flavin cofactors, FEBS J. 276 (2009) 3405–3427.

[27] N. Yamashita, T. Motoyoshi, A. Nishimura, Molecular cloning of the isoamyl alcohol oxidase-encoding gene (mreA) from *Aspergillus oryzae*, J. Biosci. Bioeng. 89 (2000) 522–527.

[28] D.P.H.M. Heuts, E.W. van Hellemond, D.B. Janssen, M.W. Fraaije, Discovery, characterization, and kinetic analysis of an alditol oxidase from *Streptomyces coelicolor*, J. Biol. Chem. 282 (2007) 20283–20291.

[29] D.P. Heuts, D.B. Janssen, M.W. Fraaije, Changing the substrate specificity of a chitooligosaccharide oxidase from *Fusarium graminearum* by model-inspired site-directed mutagenesis, FEBS Lett. 581 (2007) 4905–4909.

[30] M.W. Fraaije, C. Veeger, W.J. van Berkel, Substrate specificity of flavin-dependent vanillyl-alcohol oxidase from *Penicillium simplicissimum*. Evidence for the production of 4-hydroxycinnamyl alcohols from 4-allylphenols., Eur. J. Biochem. 234 (1995) 271–277.

[31] M.W. Fraaije, R.H. van den Heuvel, J.C. Roelofs, W.J. van Berkel, Kinetic mechanism of vanillyl-alcohol oxidase with short-chain 4-alkylphenols., Eur. J. Biochem. 253 (1998) 712–719.

[32] N.J. Turner, Enantioselective oxidation of C-O and C-N bonds using oxidases., Chem. Rev. 111 (2011) 4073–87.

[33] R.T. Winter, M.W. Fraaije, Applications of Flavoprotein Oxidases in Organic Synthesis: Novel Reactivities that Go Beyond Amine and Alcohol Oxidations, Curr. Org. Chem. 16 (2012) 2542–2550.

[34] K. Decker, H. Bleeg, Induction and purification of stereospecific nicotine oxidizing enzymes from *Arthrobacter oxidans*, Biochim. Biophys. Acta (BBA)-Enzymology Biol. Oxid. 105 (1965) 313–324.

[35] K. Decker, V.D. Dai, Mechanism and Specificity of L- and D-6-Hydroxynicotine Oxidase, European J. Biochem 3 (1967) 132–138.

[36] C. Binda, P. Newton-Vinson, F. Hubálek, D.E. Edmondson, A. Mattevi, Structure of human monoamine oxidase B, a drug target for the treatment of neurological disorders., Nat. Struct. Biol. 9 (2002) 22–6.

[37] L. De Colibus, M. Li, C. Binda, A. Lustig, D.E. Edmondson, A. Mattevi, Three-dimensional structure of human monoamine oxidase A (MAO A): relation to the structures of rat MAO A and human MAO B, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 12684–12689.

[38] D.E. Edmondson, C. Binda, J. Wang, A.K. Upadhyay, A. Mattevi, Molecular and mechanistic properties of the membrane-bound mitochondrial monoamine oxidases., Biochemistry. 48 (2009) 4220–30.

[39] L. Pollegioni, G. Molla, S. Sacchi, E. Rosini, R. Verga, M.S. Pilone, Properties and applications of microbial D-amino acid oxidases: current state and perspectives, Appl. Microbiol. Biotechnol. 78 (2008) 1–16.

[40] T.M. Kutchan, H. Dittrich, Characterization and mechanism of the berberine bridge enzyme, a covalently flavinylated oxidase of benzophenanthridine alkaloid biosynthesis in plants., J. Biol. Chem. 270 (1995) 24475–81.

[41] A. Winkler, F. Hartner, T.M. Kutchan, A. Glieder, P. Macheroux, Biochemical evidence that berberine bridge enzyme belongs to a novel family of flavoproteins containing a bi-covalently attached FAD cofactor, J. Biol. Chem. 281 (2006) 21276–21285.

[42] W.J. van Berkel, N.M. Kamerbeek, M.W. Fraaije, Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts, J. Biotechnol. 124 (2006) 670–689.

[43] M.W. Fraaije, N.M. Kamerbeek, W.J.H. Van Berkel, D.B. Janssen, Identification of a Baeyer-Villiger monooxygenase sequence motif, FEBS Lett. 518 (2002) 43–47.

[44] D.M. Ziegler, Flavin-containing monooxygenases: Enzymes adapted for multisubstrate specificity, Trends Pharmacol. Sci. 11 (1990) 321–324.

[45] C.T. Dolphin, A. Janmohamed, R.L. Smith, E.A. Shephard, I.R. Phillips, Missense mutation in flavin-containing mono-oxygenase 3 gene, FMO3, underlies fish-odour syndrome., Nat. Genet. 17 (1997) 491–494.

[46] R.N. Hines, K.A. Hopp, J. Franco, K. Saeian, F.P. Begun, Alternative processing of the human FMO6 gene renders transcripts incapable of encoding a functional flavin-containing monooxygenase., Mol. Pharmacol. 62 (2002) 320–325.

[47] A. Rioz-Martínez, M. Kopacz, G. de Gonzalo, D.E. Torres Pazmiño, V. Gotor, M.W. Fraaije, Exploring the biocatalytic scope of a bacterial flavin-containing monooxygenase., Org. Biomol. Chem. 9 (2011) 1337–1341.

[48] A. Riebel, G. De Gonzalo, M.W. Fraaije, Expanding the biocatalytic toolbox of flavoprotein monooxygenases from *Rhodococcus jostii* RHA1, J. Mol. Catal. B Enzym. 88 (2013) 20–25.

[49] A. Riebel, M.J. Fink, M.D. Mihovilovic, M.W. Fraaije, Type II Flavin-Containing Monooxygenases: A New Class of Biocatalysts that Harbors Baeyer-Villiger Monooxygenases with a Relaxed Coenzyme Specificity, ChemCatChem. (2013) n/a–n/a.

[50] M. Stehr, H. Diekmann, L. Smau, O. Seth, S. Ghisla, M. Singh, et al., A hydrophobic sequence motif common to N-hydroxylating enzymes, Trends Biochem. Sci. 23 (1998) 56–57.

[51] W.H. Walker, T.P. Singer, S. Ghisla, P. Hemmerich, Studies on succinate dehydrogenase. 8 -Histidyl-FAD as the active center of succinate dehydrogenase, Eur. J. Biochem. 26 (1972) 279–289.

[52] E.B. Kearney, T.P. Singer, On the prosthetic group of succinic dehydrogenase, Biochim. Biophys. Acta. 17 (1955) 596–597.

[53] E. de Jong, W.J.H. van Berkel, R.P. van der Zwan, J.A. de Bont, Purification and characterization of vanillyl-alcohol oxidase from *Penicillim simplicissicum*. A novel aromatic alcohol oxidase containing covalently bound FAD., Eur. J. Biochem. 208 (1992) 651–657.

[54] B.P. Zhou, D.A. Lewis, S.W. Kwan, C.W. Abell, Flavinylation of monoamine oxidase B, J. Biol. Chem. 270 (1995) 23653–23660.

[55] A. Hassan-Abdallah, R.C. Bruckner, G. Zhao, M.S. Jorns, Biosynthesis of covalently bound flavin: isolation and *in vitro* flavinylation of the monomeric sarcosine oxidase apoprotein, Biochemistry. 44 (2005) 6452–6462.

[56] N.S. Scrutton, L.C. Packman, F.S. Mathews, R.J. Rohlfs, R. Hille, Assembly of redox centers in the trimethylamine dehydrogenase of bacterium W3A1. Properties of the wild-type enzyme and a C30A mutant expressed from a cloned gene in *Escherichia coli.*, J. Biol. Chem. 269 (1994) 13942–13950.

[57] C.H. Huang, W.L. Lai, M.H. Lee, C.J. Chen, A. Vasella, Y.C. Tsai, et al., Crystal structure of glucooligosaccharide oxidase from *Acremonium strictum*, J. Biol. Chem. 280 (2005) 38831–38838.

[58] I. Alexeev, A. Sultana, P. Mäntsälä, J. Niemi, G. Schneider, Aclacinomycin oxidoreductase (AknOx) from the biosynthetic pathway of the antibiotic aclacinomycin is an unusual flavoenzyme with a dual active site, Proc. Natl. Acad. Sci. 104 (2007) 6170.

[59] X. Lu, D. Nikolic, D.J. Mitchell, R.B. Van Breemen, J.A. Mersfelder, R. Hille, et al., A mechanism for substrate-induced formation of 6-hydroxyflavin mononucleotide catalyzed by C30A trimethylamine dehydrogenase, Bioorganic Med. Chem. Lett. 13 (2003) 4129–4132.

[60] J. Kim, J.H. Fuller, V. Kuusk, L. Cunane, Z.W. Chen, F.S. Mathews, et al., The cytochrome subunit is necessary for covalent FAD attachment to the flavoprotein subunit of p-cresol methylhydroxylase, J. Biol. Chem. 270 (1995) 31202–31209.

[61] R. Brandschs, V. Bichler, Autoflavinylation of apo6-hydroxy-D-nicotine Oxidase, J. Biol. Chem. 266 (1991) 19056–19062.

[62] P. Trickey, M.A. Wagner, M.S. Jorns, F.S. Mathews, Monomeric sarcosine oxidase: structure of a covalently flavinylated amine oxidizing enzyme, Structure. 7 (1999) 331–345.

[63] J.W. Koetter, G.E. Schulz, Crystal structure of 6-hydroxy-D-nicotine oxidase from *Arthrobacter nicotinovorans*, J. Mol. Biol. 352 (2005) 418–428.

[64] J. Jin, H. Mazon, R.H. van den Heuvel, A.J. Heck, D.B. Janssen, M.W. Fraaije, Covalent flavinylation of vanillyl-alcohol oxidase is an autocatalytic process, FEBS J. 275 (2008) 5191–5200.

[65] I. Efimov, W.S. McIntire, A study of the spectral and redox properties and covalent flavinylation of the flavoprotein component of p-cresol methylhydroxylase reconstituted with FAD analogues, Biochemistry. 43 (2004) 10532–10546.

[66] D. Heuts, R. Winter, G. Damsma, D. Janssen, M. Fraaije, The role of double covalent flavin binding in chito-oligosaccharide oxidase from *Fusarium graminearum*, Biochem. J. 413 (2008) 175–183.

[67] C.-H. Huang, A. Winkler, C.-L. Chen, W.-L. Lai, Y.-C. Tsai, P. Macheroux, et al., Functional roles of the 6-*S*-cysteinyl, 8alpha-*N*1-histidyl FAD in glucooligosaccharide oxidase from *Acremonium strictum*., J. Biol. Chem. 283 (2008) 30990–6.

[68] S. Ghisla, V. Massey, Mechanisms of flavoprotein-catalyzed reactions, Eur. J. Biochem. 181 (1989) 1–17.

[69] M. Li, C. Binda, A. Mattevi, D.E. Edmondson, Functional role of the "aromatic cage" in human monoamine oxidase B: structures and catalytic properties of Tyr435 mutant proteins, Biochemistry. 45 (2006) 4775–4784.

[70] S.S. Erdem, Ö. Karahan, Ö. Yıldız, K. Yelekçi, A computational study on the amine-oxidation mechanism of monoamine oxidase: insight into the polar nucleophilic mechanism, Org. Biomol. Chem. 4 (2006) 646–658.

[71] M.A. Akyüz, S.S. Erdem, D.E. Edmondson, The aromatic cage in the active site of monoamine oxidase B: effect on the structural and electronic properties of bound benzylamine and p-nitrobenzylamine, J. Neural Transm. 114 (2007) 693–698.

[72] E.C. Ralph, J.S. Hirschi, M.A. Anderson, W.W. Cleland, D.A. Singleton, P.F. Fitzpatrick, Insights into the mechanism of flavoprotein-catalyzed amine oxidation from nitrogen isotope effects on the reaction of N-methyltryptophan oxidase, Biochemistry. 46 (2007) 7655–7664.

[73] C.W.M. Kay, H. El Mkami, G. Molla, L. Pollegioni, R.R. Ramsay, Characterization of the covalently bound anionic flavin radical in monoamine oxidase A by electron paramagnetic resonance, J. Am. Chem. Soc. 129 (2007) 16091–16097.

[74] S.E.J. Rigby, R.M.G. Hynson, R.R. Ramsay, A.W. Munro, N.S. Scrutton, A stable tyrosyl radical in monoamine oxidase A, J. Biol. Chem. 280 (2005) 4627–4631.

[75] L.B. Hersh, M.S. Jorns, Use of 5-deazaFAD to study hydrogen transfer in the D-amino acid oxidase reaction., J. Biol. Chem. 250 (1975) 8728–8734.

[76] P.F. Fitzpatrick, Oxidation of amines by flavoproteins, Arch. Biochem. Biophys. 493 (2010) 13–25.

[77] A. Tilocca, A. Gamba, M.A. Vanoni, E. Fois, First-principles molecular dynamics investigation of the D-amino acid oxidative half-reaction catalyzed by the flavoenzyme D-amino acid oxidase., Biochemistry. 41 (2002) 14111–14121.

[78] N.S. Sampson, A. Vrielink, Cholesterol oxidases: a study of nature's approach to protein design., Acc. Chem. Res. 36 (2003) 713–722.

[79] S. Macmillar, D.E. Edmondson, O. Matsson, Nitrogen Kinetic Isotope Effects for the Monoamine Oxidase B-Catalyzed Oxidation of Benzylamine and (1,1-(2)H(2))Benzylamine: Nitrogen Rehybridization and CH Bond Cleavage Are Not Concerted., J. Am. Chem. Soc. 133 (2011) 12319–12321.

[80] B. Karasulu, M. Patil, W. Thiel, Amine oxidation mediated by lysine-specific demethylase 1: quantum mechanics/molecular mechanics insights into mechanism and role of lysine 661., J. Am. Chem. Soc. 135 (2013) 13400–13.

[81] V. Massey, Activation of Molecular Oxygen by Flavins and Flavoproteins, J. Biol. Chem. 269 (1994) 22459–22462.

[82] J.P. Roth, J.P. Klinman, Catalysis of electron transfer during activation of O_2 by the flavoprotein glucose oxidase, Proc. Natl. Acad. Sci. 100 (2003) 62–67.

[83] C. Kemal, T.W. Chan, T.C. Bruice, Reaction of $3O_2$ with dihydroflavins. 1. N3, 5-Dimethyl-1, 5-dihydrolumiflavin and 1, 5-dihydroisoalloxazines, J. Am. Chem. Soc. 99 (1977) 7272–7286.

[84] V. Massey, The reactivity of oxygen with flavoproteins, Int. Congr. Ser. 1233 (2002) 3–11.

[85] A. Mattevi, To be or not to be an oxidase: challenging the oxygen reactivity of flavoenzymes, Trends Biochem. Sci. 31 (2006) 276–283.

[86] P.A. Frey, A.D. Hegeman, Enzymatic Reaction Mechanisms, Oxford University Press, 2007

CHAPTER 2

ENZYME ENGINEERING BY COFACTOR REDESIGN

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ABSTRACT

Many enzymes make use of one or more cofactors such as flavin, heme or metal ions. These cofactors have been acquired by enzymes in order to broaden the chemical possibilities of amino acids. Natural cofactors can be divided into inorganic and organic cofactors and they are mostly used by oxidoreductases. Iron is the most popular inorganic cofactor and it is most probably due to its relatively high ambient concentrations before the increase of oxygen concentration in the atmosphere. Furthermore, iron is the only transition metal which redox potential can be easily modulated by the environment. Organic cofactors vary a lot in their structure and chemical properties. Three most common redox organic cofactors are nicotinamide (NAD(P)), heme and flavin. NAD(P) plays a central role in all living cells metabolism and, therefore, many enzymes depend on this cofactor. Many modified nicotinamide cofactors have been synthesized and their chemical potential with different enzymes has been evaluated. Heme can be found in proteins with various functions, including oxygen- and electron-storage and transport, as well as catalysis. Intensive heme engineering has been performed in order to understand fundamental functions of heme proteins, but also to investigate their potential biocatalytic applications as "artificial metalloenzymes". In order to produce them, modified hemes have been reconstituted to protein scaffolds like myoglobins and albumins. The flavin cofactor is chemically diverse and is involved in a variety of redox and non-redox reactions. Engineering of the flavin cofactor included its covalent attachment to a protein scaffold, modifications in the isoalloxazine ring and reconstitution to a flavoprotein in a holo form or to a non-flavoprotein scaffold. The purpose for these studies was to understand the fundamental function and features of flavin-containing enzymes, but also to produce biocatalysts with new catalytic properties.

INTRODUCTION

Enzymes catalyse a wide range of chemical reactions in Nature, for which helper molecules – cofactors – are often employed. Cofactors are non-protein small molecules or atoms that are required in the active site of enzymes and are directly involved in catalysis. Cofactors thus extend the scope of chemistry in Nature beyond what is feasible when using only amino acids. Although more than half of all known enzymes use such "helper molecules" [1] the actual number of natural cofactors is, nevertheless, limited. In order to broaden the catalytic potential of enzymes, recent protein engineering approaches have been expanded to develop, and subsequently employ, enzymes containing redesigned cofactors.

It is assumed that many natural organic cofactors have evolved from ribozymes, which are catalytic RNA molecules. The discovery of RNA-based catalysis brought an end to the idea that enzymes are the only natural catalytic machineries, and placed RNA before DNA and proteins in the evolution of life [2,3] It is hypothesized that around four billion years ago, in the so-called "RNA world," a collection of RNAs or RNA-like molecules could function as a carrier of genetic information and as a catalyst of essential chemical reactions [4-6]. It is interesting to note that some of the most common contemporary enzyme cofactors, such as nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD), are derivatives of ribonucleotides. Furthermore, it has been found that several of the most ubiquitous cofactors can be targets for riboswitches [7-17], which are functional RNA molecules able to directly sense and regulate levels of cellular metabolites [18]. Therefore, it is speculated that cofactors were actually first utilized by ribozymes. For example, it has been proposed that NAD^+ and $NADP^+$ were adopted by ribozymes as redox cofactors [19]. Metal ions – especially divalent cations – have also been shown to facilitate the folding of ribozymes in order to form an active site and to stabilize the transition state [20,21].

As it seems quite likely that ribozymes were able to carry out a variety of important reactions, the question might be asked as to why – and how – did proteinogenic enzymes take over their functions? Clearly, the 20 common amino acids can offer any catalytic machinery a much more diverse set of functional groups than can four RNA nucleotides. Yet, the generally accepted answer – that proteins are catalytically more versatile than nucleic acids – is not quite sufficient, as evolved proteins brought advantages only in the later stages of development [22]. According

to a proposal by Szathmáry, amino acids were initially adopted by ribozymes as cofactors. For example, the chemical advantage of using lysine as a cofactor is that it carries a positive charge at neutral pH, and it can act as a general base, assisting a water-mediated nucleophilic attack [23]. Moreover, amino acid cofactors could bind reversibly to the specific sequences of ribozymes through conventional base-pairing, forming so-called "coding coenzyme handles" [22,24,25]. The amino acids could later be linked to polypeptides, while the handles turned into adaptors (transfer RNA; tRNA) such that many ribozymes lost their original enzymatic activity and became messenger RNA (mRNA) molecules, which ultimately evolved into the translational system. In this way, the coded protein enzymes could provide specific substrate binding sites that were not available in ribozymes.

In this Chapter, an overview is provided of the occurrence and role of natural enzyme cofactors. In addition, approaches to cofactor redesign are discussed, with emphasis being placed on the most frequently encountered redox cofactors, namely nicotinamide, flavin and heme.

NATURAL COFACTORS: TYPES, OCURRANCE AND CHEMISTRY

According to the International Union of Pure and Applied Chemistry (IUPAC) [26], cofactors are "…organic molecules… or ions… that are required by an enzyme [for] its activity". The definition also explains that "…a cofactor binds with its associated protein (apoenzyme), which is functionally inactive, to form the active enzyme (holoenzyme)." However, quite often this definition is further refined by a requirement that a cofactor must be present in the catalytic site, which excludes examples of constitutive allosteric regulation [27]. Furthermore, the IUPAC definition divides cofactors into two types: those attached to an enzyme loosely (coenzymes), and those attached tightly (prosthetic group). When a cofactor is incorporated into a cofactor-dependent enzyme, the reactivity of the cofactor is influenced by the protein environment. Hence, a holoenzyme can attain substrate and product selectivities and rates and yields that would not be possible for either the apoenzyme or cofactor alone.

When examining their chemical composition, cofactors can be divided into two main groups:

- Inorganic cofactors, such as metal ions and iron-sulfur clusters [28,29].
- Organic cofactors, such as flavin and heme cofactors [30,31].

In general, metal ions are most active in the area of electrostatic stabilization and are more potent than amino acids – but not than organic cofactors – in pair and single-electron shuttling. Organic cofactors are able to support a wide range of reaction types, with many organic cofactors being involved in electron-transfer processes. This is in agreement with the fact that 80% of all oxidoreductases employ an organic cofactor.

A given cofactor can perform a mechanistically identical reaction in various enzyme scaffolds (e.g., NAD for hydride transfer), or its mechanism of action can depend on the protein (e.g., S-adenosylmethionine as a methyl donor or radical generator) [27]. It is also possible that some cofactors occur only in one enzyme, such as dipyromethane in hydroxymethylbilane synthase (see Table 2.1), while some enzymes or enzyme complexes employ several cofactors. For example, formylmethanofuran dehydrogenase is composed of three subunits containing, respectively, a flavin, an iron-sulfur cluster, and two molybdopterin guanine dinucleotide cofactors coordinated to a tungstate [32].

INORGANIC COFACTORS

Information concerning the properties and roles of metal ions involved in catalysis of metal-dependent enzymes is available in the Metal-MACiE database [29]. An analysis of specific chemical functions of metal cofactors, based on the database and structural information, has been summarized by Andreini et al. [28], according to whom oxidoreductases (EC 1) mostly use metals, especially iron, as their redox centres. When a metal is used as a redox catalyst, it is usually bound to an organic cofactor (e.g., iron in heme), but this is not the case in non-redox catalysis. Metal ions in transferases (EC 2) are mostly electrostatic stabilizers and/or activators, and increase the electrophilicity of the substrate so that nucleophilic substitution/addition becomes possible. Transferases most often utilize magnesium as their cofactors.

In the case of hydrolases (EC 3) and lyases (EC 4), no redox-active metals are employed. Rather, metals in hydrolases either activate the substrate or stabilize the electrostatic charge, with zinc appearing to be the most suitable metal for both functions. The role of metals in lyases is to induce proton transfer from the substrate; magnesium and zinc are each used for this purpose, based on their ability to enhance the acidity of the substrate.

Isomerases (EC 5) can be divided into two groups: (i) cobalamin-dependent, generating radical species; and (ii) metal ion-containing isomerases. Members of the latter enzyme group – the metal-dependent ligases (EC 6) – prefer magnesium to perform an S_N2 nucleophilic attack of the substrate on ATP (or an analogue).

The domination of iron in redox systems is most likely the consequence of relatively high ambient concentrations of Fe(II), before atmospheric oxygen levels were increased due to the development of photosynthesis, and which resulted in the formation of insoluble $Fe(OH)_3$. The redox potential of iron also depends very much on the protein microenvironment, which means it can be easily modulated [33], though this is not the case with other transition metals. In iron-containing enzymes the metal can be found in either mononuclear or binuclear sites [34].

The function of mononuclear iron in enzymes is to activate dioxygen with a high-spin ferrous (Fe[II]) site or to activate substrates with a high-spin ferric (Fe[III]) site [34]. The process of dioxygen activation is kinetically hindered, however, because it requires singlet oxygen, whereas atmospheric O_2 exists mostly as triplet oxygen. Iron, as well as copper, is able to overcome this kinetic barrier because it can form a coordination bond, which is directly followed by the transfer of a second electron to the oxygen. Reduction is achieved by an auxiliary redox active cofactor (α-ketoglutarate-dependent dioxygenases, pterin-dependent hydroxylases, and Rieske dioxygenases) or a redox-active substrate, which supplies the necessary reducing equivalents for oxygen activation (e.g., extradiol-cleaving catechol dioxygenase [35]).

Substrate-activating enzymes include the lipoxygenases and the intradiol dioxygenases. In binuclear iron enzymes, a diferrous site is involved in the reversible binding of oxygen (hemerythin [36]) and oxygen activation (ribonucleotide reductase [37-40], methane monooxygenase [41-43] and Δ^9 desaturase [44]). In the case of oxygen activation, however, the second electron required for this process is derived from the second metal located within, or in close proximity to, the catalytic site. As a representative example, in methane monooxygenase, dioxygen binds to the Fe(II)–Fe(II) cluster to enable the ready formation of a Fe(III)–Fe(III)-peroxo-adduct [45,46].

Another group of small inorganic cofactors includes the iron-sulfur (Fe–S) clusters, which are thought to be among the earliest catalysts in the evolution of molecules [47,48]. (Fe–S) species are built from iron and bridging sulfur elements at various molar ratios [49], with the rhombic (2Fe-2S) and the cubane

(4Fe-4S) types being most common, while (3Fe-4S) and (8Fe-7S) have also been identified. In most proteins, the iron elements are coordinated to cysteine residues, although histidine, serine, aspartic acid residues, or back-bone amides can also act as ligands [50-53]. These can serve in biological electron transport due to their ability to delocalize electron density over Fe and S atoms [54-56], and consequently, they are major components in the photosynthetic and respiratory electron transport chains. Moreover, they are able to transport electrons in many membrane-bound and soluble redox enzymes, and constitute the redox-active centres in ferredoxins, one of the largest classes of mobile electron carriers in biology [57]. Whereas the (Fe–S) species are one-electron carriers, the double-cubane (8Fe-7S) cluster, which is found only in nitrogenases, has the potential to act as a two-electron carrier [58].

ORGANIC COFACTORS

An overview of the molecular properties of organic cofactors has been produced by Fischer et al. [27], while details are also available in the CoFactor database [30,31] (see Table 2.1). Organic cofactors vary the most in terms of polarity and size, and less in flexibility. The porphyrin-like molecules, such as heme, B12, siroheme, cofactor 430, menaquinone 7 (MQ7), and ubiquinone 10 (U10), are larger and more hydrophobic than average. The dinucleotide cofactors (NAD, NADP, FAD, coenzyme A, and molybdopterin guanine dinucleotide) form the second group of cofactors, which are rich in heteroatoms and are of medium to high molecular weight. The remaining cofactors are smaller in size, with a lower hydrogen-bonding potential, and are a relatively diverse group.

Table 2.1. Overview of known organic enzyme cofactors (based on the CoFactor database (31), October 2011).

Cofactor	Molecular function	% in EC classes						total
		1	2	3	4	5	6	
Adenosylcobalamin	molecular rearrangements; methylations; dehalogenations	7	13	7	27	47	0	15
Ascorbic acid	antioxidant defence, particularly *via* the ascorbate/glutathione cycle	95	0	0	0	5	0	20
Biopterin	one- or two-electron transfers; OH-group transfer for aromatic amino acids	100	0	0	0	0	0	6
Biotin	transfer CO_2 (or C_2-units) from one active site of an enzyme complex to another	0	9	0	27	0	64	11
Coenzyme A (CoA)	the transfer of acyl group	10	71	6	1	0	12	237
Coenzyme B	a base in the methanogenesis reaction	0	100	0	0	0	0	1
Coenzyme M	a methyl group donor in the methanogenesis reaction	0	100	0	0	0	0	1
Dipyrromethane (DPM)	a nucleophile and acts as a seed for heme synthesis	0	100	0	0	0	0	1
Factor F430	binds and releases the methyl leaving group from coenzyme M and catalyses the initiation and termination of a radical reaction in methanogenesis	0	100	0	0	0	0	1
Flavin adenine dinucleotide (FAD)	one and two electron transfer reactions; radical reactions; photoreceptor-induced reactions	92	3	1	3	1	0	235
Flavin mononucleotide (FMN)	both one and two electron transfer reactions; radical reactions	79	9	5	5	2	0	43
Glutathione (GSH)	counteracts oxidative stress	51	14	14	11	6	3	35
Heme	source of electrons; catalyses oxygenation of substrates: R-H → R-OH (P450); also present in other oxidative enzymes	95	0	1	3	2	0	129

Lipoic acid	allows the reactant to access three different catalytic sites (swinging arm); carries the substrate	83	17	0	0	0	0	6
Menaquinone (vitamin K)	formation of γ-carboxyglutamyl (Gla) residues from specific glutamate residues in certain proteins	0	0	0	100	0	0	1
MIO (4-methylideneimidazole-5-one)	strong electrophile, abstracts a relatively non-acidic hydrogen atom	0	0	0	75	25	0	4
Molybdopterin	electron transfer	100	0	0	0	0	0	14
Nicotinamide-adenine dinucleotide (phosphate) (NAD(P))	hydride transfers	95	2	1	1	1	0	745
Orthoquinones (LTQ, TTQ, CTQ)	two-electron two-proton reaction (dehydrogenases)	100	0	0	0	0	0	2
Phospho-pantetheine	bind substrate and then function as an agile arm to bring the (growing) substrate from one active site of an multi-domain complex to the next	25	50	12.5	12.5	0	0	8
Pyridoxal 5'-phosphate (PLP)	de/transamination, decarboxylation, racemization, aldol condensation, α,β-elimination and β,γ-elimination of amino acids, and amine oxidation	1	53	1	37	8	0	139
Pyrroloquinoline quinone (PQQ)	electron transfers	100	0	0	0	0	0	9
S-adenosylmethionine	methyl-donor; radical reactions using the adenosyl-radical; amino donor	4	89	1	6	0	0	70
Tetrahydrofolic acid (THF)	transports and donates C_1	0	83	0	17	0	0	6
Thiamine diphosphate (ThDP)	formation and cleavage of C-S, C-N, C-O and the chemically challenging C-C bonds using the enamine intermediate carbanion	30	27	3	40	0	0	30
Topaquinone (TPQ)	oxidation of amines	100	0	0	0	0	0	2
Ubiquinone (CoQ)	electron transport in the respiratory chain	100	0	0	0	0	0	7
	Variance in cofactor usage in each enzyme class (number of cofactor types per EC)	19	17	11	15	9	4	

For each type of cofactor there is a bias of its occurrence in enzymes of a particular EC class. The distribution of a certain cofactor, as well as the number of enzymes employing it, can differ significantly (Table 2.1). For example, coenzyme A (CoA) is used by a large number of enzymes (n = 237) from many EC classes, while NAD is found almost exclusively in oxido reductases (EC 1). In contrast, some cofactors are used by only a few enzymes from one enzyme class, such as biopterin (six oxidoreductases) or menaquinone (one lyase).

The distribution of organic and inorganic cofactors among all enzyme classes is as follows. The oxidoreductases (EC 1) are an enzyme class that depend mostly on organic cofactors, while hydrolases (EC 3) use such cofactors very rarely. Approximately one-third of enzymes from the remaining enzyme classes – the transferases (EC 2), lyases (EC 4), isomerases (EC 5), and ligases (EC 6) – employ organic cofactors.

This distribution can be explained by the complexity of the reactions that the enzymes catalyse. For example, the catalytic mechanism of hydrolases is relatively simple, and these enzymes quite often use metal ions as cofactors, as they can activate water for an initial nucleophilic attack [27]. Redox functions are rather unique to organic cofactors, including hydride, proton, single-electron and electron-pair transfer, and even covalent catalysis [27].

One organic cofactor, adenosine 5'-triphosphate (ATP), was not included in the CoFactor database, and thus is not described in Table 2.1. ATP typically serves as a coenzyme, facilitating phosphate group transfer. The hydrolysis of ATP, leading to the formation of ADP and inorganic phosphate, yields energy that is typically used to drive a thermodynamically unfavourable reaction. ATP also serves as a building block for RNA. As a coenzyme, ATP is used primarily by kinases (EC 2.7) in order to phosphorylate alcohols, and by some ligases (EC 6.5) in order to activate carboxyl groups, forming acyl-phosphate intermediates.

REDOX COFACTORS

Redox enzymes (oxidoreductases) catalyse reduction and oxidation reactions. As noted above, the chemical functionality of amino acids is limited, and for this reason redox enzymes typically need to employ redox-active transition metals or organic cofactors. Oxidoreductases can be divided into several groups, according

to the reaction that they catalyse and to the cofactor they use (Fig. 2.1). In many cases, oxidoreductases rely on a redox coenzyme such as NAD(P), which acts as an electron-shuttling cosubstrate that has to be regenerated. The redox reaction can also be coupled with the reduction of hydrogen peroxide to water (peroxidases), the reduction of oxygen to hydrogen peroxide or water (oxidases), and the reduction and incorporation of oxygen (some dioxygenases). The characteristics of three of the most common redox cofactors, nicotinamide, heme, and flavin, and related cofactor redesign studies will be reviewed in the following sections.

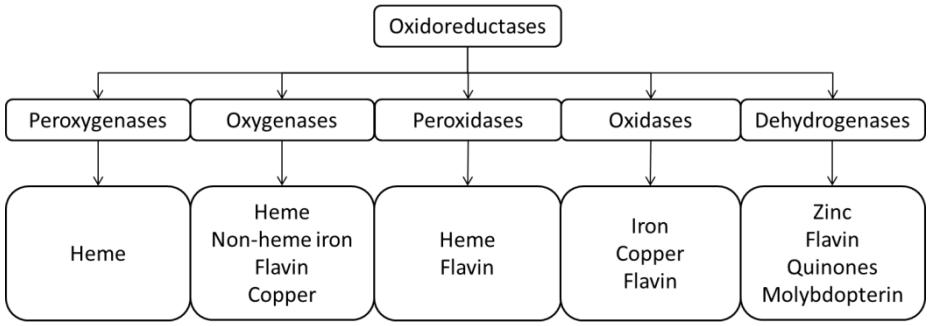


Figure 2.1. Classification of oxidoreductases and their respective most commonly found cofactors.

Nicotine cofactor engineering

Nicotinamide adenine dinucleotide (NAD) is the central redox cofactor in all living cells. It serves as hydride-mediator in biocatalytic redox mechanisms, shuttling between its oxidized form, $NAD(P)^+$, and its reduced form, NAD(P)H (Fig. 2.2).

NAD exists in both a phosphorylated (NADP) and a non-phosphorylated form (NAD), with the variants being used either for catabolic or anabolic redox reactions in the living cell. Hence, most native enzymes are usually highly specific for either NAD or NADP. Since its discovery and initial characterization in the early twentieth century [59,60], investigations into modified, (semi-) synthetic NAD analogues have been conducted in approximately two "waves," with different motivations. First, during the 1950s to 1970s, attention was focused on identifying a fundamental understanding of the biological role and chemistry of NAD. Subsequently, more practical implementations were highlighted. In particular, it was Kaplan and coworkers who initiated the "first wave"

of pioneering experiments to elucidate the steric and electronic requirements of the nicotinamide moiety of NAD [61-66]. By using pig brain NADase (E.C. 3.2.2.5), it was found that the native nicotinamide (pyridine-3-carboxamid) moiety could be replaced by other pyridine derivatives, thus enabling investigations of the effects of such replacements on enzymatic activity (Scheme 2.1).

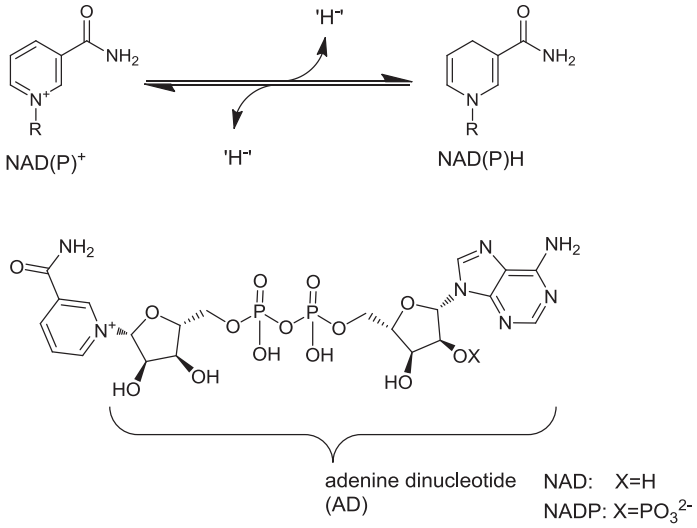
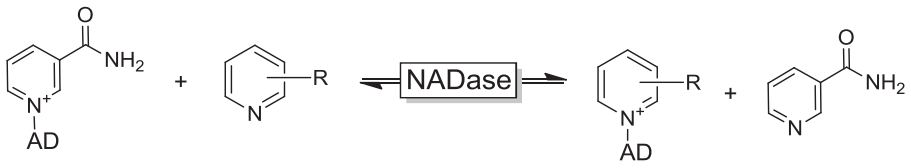


Figure 2.2. Redox chemistry of NAD(P) (upper) and general structure of NAD(P) (lower).



Scheme 2.1. Enzymatic synthesis of modified NAD analogues (mNAD) using NADase.

By using this methodology, a wide range of modified nicotinamide cofactors (mNADs) have been synthesized and evaluated for their acceptance, especially with alcohol dehydrogenases (ADHs) [61,63,64,67-73] (a detailed discussion of these modified cofactors is beyond the scope of this chapter). The majority of mNADs evaluated exhibited significantly decreased enzyme activities, as expressed by K_M and V_{max} values. In addition, mechanistic studies [69,70,74],

molecular mechanics simulations [75], and crystallographic investigations [76] were conducted to shed light on the interaction of NAD and its analogues with enzymes. In some cases, however, an increased ADH activity was also observed, amongst which 3-acetylpyridine-, 3-thioacetamide-, and 3-isobutyrylpyridine- derivatives (Fig. 2.3) exhibited significantly improved activities.

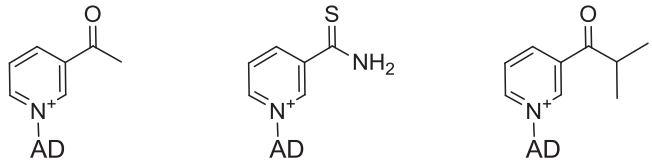


Figure 2.3. Selection of mNADs exhibiting significant activity with various ADHs.

In one very interesting study, Kazlauskas [77] examined the horse liver-ADH-catalysed oxidation of various alcohols by mNADs. In this case, electron-poor $mNAD^+$s exhibited an up to ninefold acceleration of the hydride transfer from the alcohol to $mNAD^+$ but, surprisingly, product inhibition by the corresponding aldehydes and ketones was drastically decreased. This observation could not be rationalized, however, and the same was true for the change in enantioselectivity observed upon substituting NAD with mNADs [78]. More recently, mNADs have been rediscovered as biomedical probes and agents [79-82]. For example, masked, enzymatically inactive mNADs have been synthesized which can be reactivated photochemically. Likewise, a covalent inhibition of ADHs was achieved with photoactivatable arylazide-substituted NADs [83], whereby modifications at the adenine dinucleotide moiety (AD; Fig. 2.2) generally led to a significantly decreased activity of the resulting mNAD [84-86]. An example of this was reported by Sicsic et al., who evaluated truncated NAD models such as nicotinamide mononucleotide and its dephosphorylated analogue nucleotinamide nucleoside [87,88]. In this case, the use of horse liver-ADH led to a dramatic decrease in enzyme activities which, to some extent, could be recovered by the addition of adenine.

With the emergence of White Biotechnology (i.e., the application of biotechnology to industrial processes) during the 1980s, economic issues in enzyme-mediated technologies have begun to gain importance. Indeed, the (still) high price of natural nicotinamide cofactors has triggered the development of efficient systems

to regenerate cofactors that can then be used in catalytic quantities [89]. In order to create nicotinamide cofactors that were compatible with well-known enzyme membrane reactors (EMRs), thereby enabling their use in continuous production systems, polymer-modified NADs have been developed by Kula and Wandrey [90]. As a consequence, polyethylene glycol-modified NAD (PEG-NAD) derivatives were shown to be applicable to a broad range of dehydrogenase-catalysed reactions in EMRs [90-100]. An alternative approach to utilizing fully synthetic (and hence cheaper) nicotinamide analogues was proposed by Loewe and coworkers (Fig. 2.4) [101-108], and involved linking nicotinamide covalently to triazine dyes. However, on the evaluation of these artificial NAD analogues the catalytic performance proved to be rather poor. More recently, Fish and coworkers suggested the use of N-benzyl nicotinamide derivatives (Fig. 2.4) for ADH-catalysed reductions [109] and monooxygenase-catalysed oxyfunctionalization reactions [110,111]. Unfortunately, the catalytic performance of these artificial mNADs was inferior, by several orders of magnitude, to the natural nicotinamide cofactors. Furthermore, when a flavin-dependent monooxygenase was used, a drastic increase in the uncoupling of flavin-reduction (mediated by mNAD) from product hydroxylation was observed [110]. This effect was considered due to a stabilizing H-bonding interaction between the oxidized nicotinamide cofactor and the intermediate 4a-hydroperoxyflavin [112].

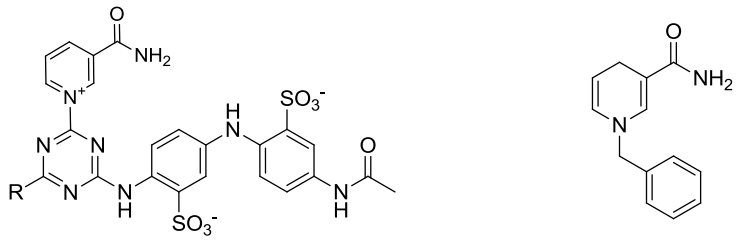


Figure 2.4. Synthetic mNADs proposed by Loewe (left) and Fish (right).

Overall, the studies of (semi-)synthetic analogues of the native nicotinamide cofactors have enriched the present understanding of the catalytic mechanism of NAD-dependent reactions. Whilst practical applications may arise from the therapeutic use of some mNADs, the promise of cell-free biocatalytic applications of oxidoreductases being cheaper by using mNADs has not yet been fulfilled.

Rather, the high specificity of wild-type oxidoreductases for their natural redox cofactor, which results in highly unfavourable enzyme kinetics with mNADs, largely outweighs any cost benefits. Nonetheless, it is conceivable that protein engineering might offer a suitable solution to this problem, and in this respect Zhao and coworkers [113] have described an interesting concept of bioorthogonal redox reactions. Due to the central role of native nicotinamide cofactors in cellular metabolism, NAD(P)-dependent whole-cell biotransformations are difficult to control and optimize. However, it was shown that modified cofactors (at the AMP moiety), together with engineered production and regeneration enzymes, could be used for redox biocatalysis. Hence, the desired redox reaction would be independent of the cellular NAD(P) metabolism, allowing straightforward optimization.

Heme cofactor engineering

In Nature, heme proteins fulfil central functions in diverse roles such as oxygen- and electron-storage and transport, as well as catalysis. Consequently, the biological importance and chemical versatility of heme proteins has triggered intensive research on "heme engineering." Besides seeking a more fundamental understanding of heme proteins, these efforts have been driven also by the potential catalytic applications of "artificial metalloenzymes." In particular, various research groups [114-119] have been inspired to combine the "best of two worlds," by incorporating chemical catalysts into biological scaffolds, such that a combination of the chemical versatility of these catalysts with typical enzyme characteristics, such as stereodifferentiation and rate enhancements, is envisaged. Whilst an exhaustive discussion of this thriving area of research is beyond the scope of this chapter, the topic has been well summarized in excellent recent reviews [114-119]. At this point, attention is focused on the (re)constitution of natural heme proteins and non-heme proteins with artificial heme derivatives.

Reconstitution of myoglobin

Myoglobin (Mb) represents an excellent model system for the evaluation of artificial heme moieties. Due to its essential role as an oxygen-storage protein it has been well characterized and reliable protocols have been prepared for the generation of apo-Mb [120,121]. For example, Hayashi and coworkers have conducted

extensive investigation into the substitution of native heme with modified, highly charged derivatives (Fig. 2.5) [122-125].

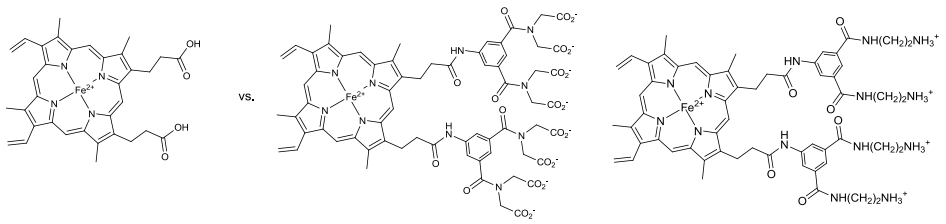


Figure 2.5. Natural heme b of Mb vs. the highly anionic and cationic derivatives.

As expected, the interaction of such a modified Mb with other proteins such as cytochrome c was significantly modulated [124,126], although the catalytic features of the artificial metalloproteins were also influenced by the modification [127]. For example, the peroxidase activity of an anionic modified Mb was greatly enhanced towards positively charged substrates such as $(Ru[NH_3]_6)^{2+}$, or phenolics such as catechol, due to favourably enhanced K_M and V_{max} values. Negatively charged substrates were, in contrast to native Mb, hardly converted at all. When combined with engineered Mb variants, peroxidation efficiencies similar to horseradish peroxidase were achieved [128], as well as improved oxyfunctionalization activities [129]. Interestingly, the reconstitution of apo-Mb with iron porphycene, a structural analogue of native heme, resulted in an Mb variant that exhibited a superior oxygen-binding capacity [130].

Due to their importance in chemical catalysis [131], it is not surprising that salen complexes have also been evaluated within the Mb scaffold. For example, Watanabe and coworkers reconstituted apo-Mb with Cr- and Mn-salophen complexes, and then evaluated the resulting hybrid catalysts for hydrogen peroxide-driven sulfoxidation reactions [132]. In this case, noteworthy enhancements in rate compared to the Mb-free catalysts and, more importantly, a significant chiral induction, were achieved (enantiomeric purities of the product up to 13% enantiomeric excess; *e.e.*). The chemoselectivity of the sulfoxidation was also influenced by the protein, as overoxidation to the (achiral) sulfone was efficiently prevented [133]. This effect was rationalized by the rather hydrophobic protein environment around the bound catalyst, which significantly hampered binding of the polar sulfoxide.

These findings were corroborated by crystallographic data [134], and this led to the development of Mb variants with increased enantiospecificities [135]. In-depth mechanistic studies also gave rise to a greater molecular understanding of the factors influencing the activity/stability/specificity of these hybrid catalysts [135,136]. The enantiospecificities achieved using these (reversible binding-based) approaches remained rather limited, but it was proposed by Lu and coworkers that this might be due to the high conformational flexibility of the Mb-bound catalyst. Hence, Mb-mutants were designed that were capable of covalently binding a modified Mn–salophene complex (Fig. 2.6) *via* newly introduced cysteines [137].

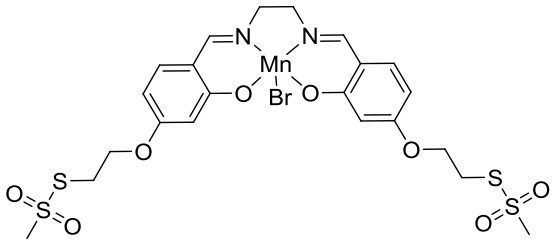


Figure 2.6. Modified Mn-salophene complex capable of double covalent linkage to a double Cys-Mb mutant (L72C/Y103C-Mb).

Indeed, the enantiospecificity was increased from 12% *e.e.* to 51% *e.e.* from the single to double covalent attachment; in fact, in a recent study that included additional mutations, *e.e.*-values of up to 83% were achieved [138]. Besides the aforementioned Cr– and Mn–salen complexes, Fe- [139], Cu– [140], and Rh–salen complexes [141] have also been incorporated into Mb.

Besides chemical modifications at the porphyrin ring, metal exchanges have also been reported that clearly enable a drastic broadening of the chemical reactivity of the reconstituted Mbs. For example, Willner and coworkers exchanged the natural Fe(II)-heme of Mb with the Co(II)-homolog [142-144] such that, after covalent modification of the reconstituted Mb with the photosensitizer eosin, an artificial photoactivated reductase was obtained (Fig. 2.7). An efficient electron transfer from the photoexcited eosins occurred efficiently only when these were covalently attached to the reconstituted Mb. Nevertheless, in that case an intermediate Co(III)–H-species was formed that was capable of efficiently catalysing *syn*-hydrogenations of C–C-double and triple bonds.

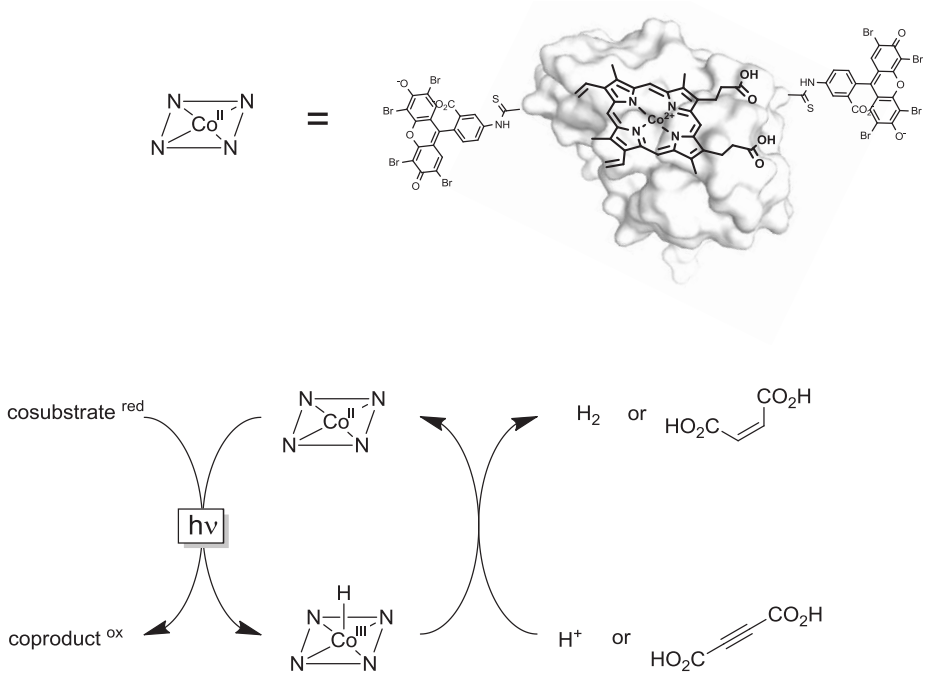


Figure 2.7. Co(II)-porphyrin-reconstituted Mb covalently modified with two eosin photosensitizers (upper) and its application in photobiocatalytic reduction of C-C multiple bonds or protons.

Artificial metalloproteins based on serum albumins

Serum albumins (SAs) represent the most abundant proteins in vertebrate blood, serving as transporters for small, hydrophobic molecules such as fatty acids, bile acids, bilirubin, and hemin [145]. In terms of hydrophobicity, the "SA hemin binding site" is comparable to the hemin binding site of Mb, which suggests a "reconstitution" of SAs with porphyrin- and salen-catalysts. Hence, it is not too surprising that SAs have been applied as chiral ligands for a range of chemical reactions [146]. During the early 1990s, Ohkubo and coworkers described the dioxygenase activity of a Mn(III)–porphyrin covalently attached to SA [147,148]. Later, Gross and coworkers systematically screened SAs from different origins combined with Fe(III)– and Mn(III)–corroles as sulfoxidation catalysts [149,150]. In particular, the Mn(III)–corrol–SA conjugate showed a high catalytic activity (up to 33 h^{-1}) and high stereospecificity (optical purities up to 74% *e.e.*) in the peroxide-driven sulfoxidation of thioanisole. Similar to previous observations

with Mbs, the SA–salen conjugate-catalysed sulfoxidation also proceeded chemoselectively to the sulfoxide [151]. Again, it was considered that the rather hydrophobic protein environment would efficiently exclude binding of the sulfoxide. Later, Reetz described a SA-supported phthalocyanine–Cu(II) complex for enantiospecific Diels-Alder reactions (Fig. 2.8) [152]. In this case, while the *endo* : *exo* ratio was largely determined by the catalyst used, the high stereoselectivity (up to 93% *e.e.*) observed for the major *endo*-diastereomer was due to the chiral protein scaffold.

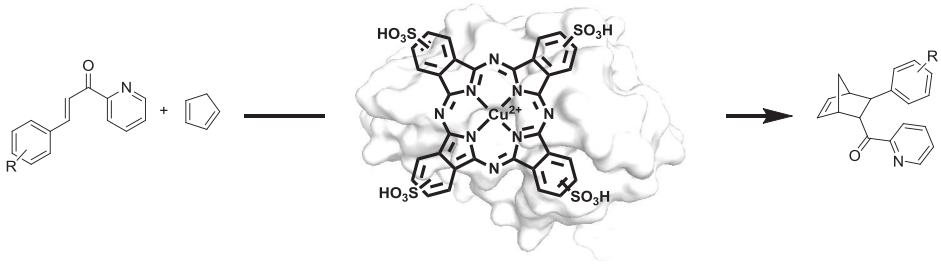


Figure 2.8. Cu(II)-phthalocyanine-BSA conjugate for enantiospecific Diels-Alder-reactions.

Flavin cofactor engineering

Cofactors containing 7,8-dimethyl-10-alkylisoalloxazine, termed "flavins," are relatively abundant in Nature and are involved in a multitude of redox and non-redox reactions. Flavin-containing proteins are extremely diverse in the reactions that they catalyse, including dehydrogenations [153], oxidations [154-156], monooxygenations [157-159], halogenations [160-162], reductions [163,164] and biological sensing [165-172]. Riboflavin (vitamin B2) is a biosynthetic precursor for the two most common flavin cofactors, namely flavin adenine dinucleotide (FAD) [173,174] and flavin mononucleotide (FMN) [175] (Fig. 2.9). It should be noted that, from a chemical point of view, FMN and FAD are not nucleotides as the bond between the ribose and isoalloxazine ring is not glycosidic. Unlike other cofactors, the reactions catalysed by flavins do not share mechanistic pathways [176]; rather, the only shared feature of flavins is the fact that the redox-active centre is always located in the isoalloxazine ring system. The amphipathic character of the system derives from three rings: the hydrophobic dimethylbenzene; the more polar pyrimidine; and joining them the central phenylenediamine ring. Electrons are

delivered to, and removed from, the flavin through this central moiety, while the pyrophosphate moiety functions as a component of molecular recognition, and the adenine ring is exploited for effective binding [177].

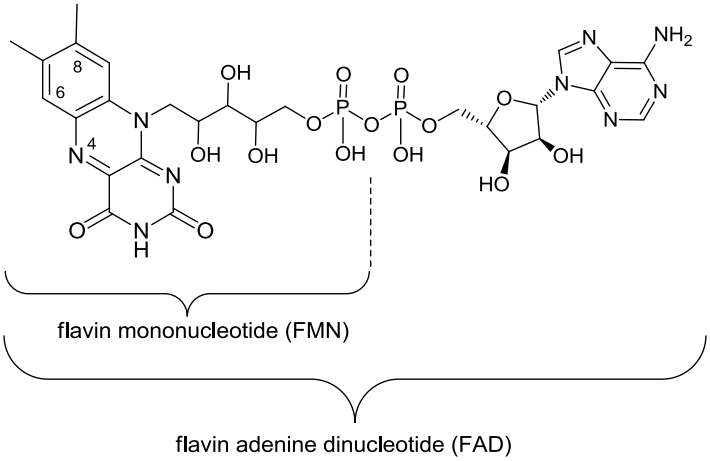


Figure 2.9. Structural formulas of the flavin cofactors FMN and FAD.

The biochemical function of flavin cofactors is based on their redox-active isoalloxazine ring system, which is able to catalyse (light-induced) either one- or two-electron transfer reactions, as well as dioxygen activation [178]. The ability to participate in one-electron transfer reactions implies that a flavin can exist in a radical form, termed a "semiquinone." Indeed, this is one of the features that set flavins apart from nicotinamide cofactors, as the semiquinone can exist in two ionic forms – neutral and anionic – which show different spectral properties [179]. The flexible redox behaviour of flavins has translated into a wide range of different flavoenzymes that are very versatile in terms of substrate acceptance and type of reaction catalysed.

In contrast to NAD(P) cofactors, flavin cofactors are usually tightly associated with the host protein. Although most flavoenzymes contain non-covalently bound FAD or FMN, some have been found to be covalently bound [180]. It appears that a covalent attachment of FMN is relatively rare when compared to FAD [181]. FMN is linked either *via* the 8α-position (to a histidine) or *via* the 6-position

(to a cysteine) of the isoalloxazine ring [182]. In the case of FAD, a covalent bond typically involves the 8α-position, which binds to N1 or N3 of a histidine, a cysteine thiol, a tyrosine hydroxyl, or an aspartate carboxyl group [182,183]. The results of recent studies have shown that, in some cases, the FAD cofactor is even bicovalently tethered *via* the 8α-position and the 6-position of the isoalloxazine ring [184]. The natural occurrence of covalent protein–flavin linkages has inspired research aimed at creating artificial covalent flavoproteins. In fact, the covalent anchoring of a flavin cofactor can result in a more stable enzyme with better or new redox properties [101-108]. The first attempts to create flavin–protein hybrids were reported back in the 1970s and 1980s [185], when a flavin group was incorporated into the hydrolytic enzyme, papain, to create a new semi-synthetic oxidoreductase. The active site cysteine residue was used to form a covalent linkage with a highly reactive brominated flavin, while the redox activity of flavopapains created in this way was determined using N-benzyl-1,4-dihydronicotinamide. One of the flavopapains showed a 50-fold rate acceleration compared to the respective free flavin, a finding rationalized by the favourable interaction of a flavin carbonyl group with papain that correctly positions the flavin cofactor in the papain active site. The importance of the exact structure of the flavin derivative attached to papain was further investigated [186] by preparing a series of flavopapains that showed widely different rate enhancements (zero to three orders of magnitude) in catalysing oxidation of the above-mentioned substrate. The differences were explained by different geometries in the active sites of the semisynthetic enzymes, where subtle differences contributed greatly to the resulting specificity.

In Nature, formation of the flavin–protein covalent bond is a post-translational, autocatalytic process (termed covalent flavinylation) that has been investigated in many flavoenzymes by preparing the apo enzyme and performing subsequent reconstitution studies *in vitro* [187-192]. Recently, it was also shown feasible to introduce a covalent linkage *in vivo* into a flavoprotein, putrescine oxidase (PuO). Originally, this bacterial amine oxidase did not contain such a covalent cofactor–protein interaction [193,194]. PuO is highly homologous to monoamine oxidase B (MAO-B) but, in contrast to MAO-B, native PuO binds FAD in a noncovalent manner. In MAO-B, the bond is formed between the C8α-methyl group of the isoalloxazine ring and a cysteine residue. Based on this observation, a Cys residue was introduced into a corresponding position in PuO, and the resulting isolated mutant protein was found to contain some covalently bound FAD (10–40%).

Two additional mutations were introduced in PuO to boost the covalent FAD binding, and the subsequently obtained triple mutant did indeed show an increased level of covalent flavin–protein bond formation [194]. These study findings supported the suggestion that covalent flavinylation depends on a correct cofactor environment [195-201] and also showed that it is feasible to turn natural noncovalent flavoproteins into the covalent variants by protein engineering. Moreover, such an approach avoids the use of highly reactive flavin analogues, which are difficult to prepare and form a covalent protein–flavin bond upon reconstitution with apo proteins *in vitro* [101-108].

Both, FMN and FAD cofactors are typically tightly bound by virtue of the tight interaction with the phosphate or ADP moieties, respectively. Consequently, it is often possible to replace a flavin cofactor with an analogue which has been modified on the isoalloxazine moiety. Similar to the initial biochemical studies performed with NAD derivatives (see above) during the 1950s to 1970s, many flavin derivatives were prepared and analysed as probes to study the role and function of flavins in flavoproteins. In fact, much of the current knowledge relating to flavoproteins originates from these flavin replacement studies (for a comprehensive review, see Ref. [202]).

The variety of modifications that have been studied in flavoproteins is illustrated in Fig. 2.10. While such flavin derivatives have been useful for elucidating the mechanistic details of how flavoenzymes function, very little attention has been focused on using flavin derivatives to create flavoproteins with novel catalytic properties. Very recently, however, such an approach proved to be successful when using alkylated flavin derivatives. It had been shown during the 1970s, that *N*5-alkylated flavins form remarkably stable 4a-peroxyflavins upon reaction with hydrogen peroxide, and thus represent powerful oxidizing catalysts [203-205]. Recently, a selection of *N*5-alkylated riboflavin and lumiflavin derivatives was prepared and used to replace the riboflavin in riboflavin-binding protein (RfBP), an avian protein that serves as a vitamin storage protein in chicken eggs but displays no catalytic activity. However, when the natural riboflavin was replaced with alkylated flavins, an artificial enzyme was created that was able to perform peroxide-driven enantioselective sulfoxidations [206]. Unfortunately, the newly created "flavozyme" proved to be a far-from-perfect biocatalyst, and could not be optimized by protein engineering (RfBP cannot be produced recombinantly [207]). Consequently, more suitable apo flavoproteins should be targeted for

generating improved variants of these artificial flavin-containing peroxygenases. Based on the outcome of recently developed methods of flavoprotein periplasmic expression in *Escherichia coli*, as well as the availability of riboflavin auxotrophic strains, the direct expression of apo proteins capable of binding externally added flavin derivatives should soon be possible [192,208]. Notably, this would open the door to the creation of not only new biocatalysts but also of recombinant organisms displaying bioorthogonal metabolic pathways.

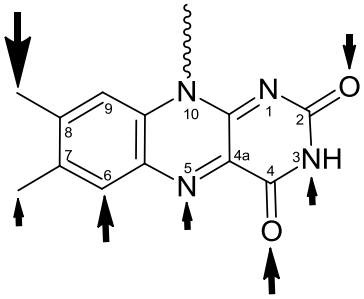


Figure 2.10. Reported sites of flavin modifications. The location and size of the arrows indicate the position and frequency of modifications of flavins that have been incorporated in flavoproteins [202].

A rather newly proposed role for redesigned flavins is based on the use of reactive flavin derivatives to develop a new drug targeting system, or to create noninvasive molecular imaging biomarkers. The strategy is based on a (as yet unknown) mechanism of riboflavin transport into cells [209], which appears to be related to the human riboflavin carrier protein. The transport protein has been shown to be highly upregulated in several types of tumour cell [210-213], most likely due to the relatively high energy demands of cancer cells that require the activation of numerous catabolic enzymes, many of which contain a flavin cofactor. This selective import of flavins makes the targeting of tumour cells possible with the use of flavin derivatives. Very recently, when riboflavin was conjugated with a novel type of (polyamidoamine) dendrimer, the result was a series of nontoxic, nonimmunogenic nanoparticles with an extended duration of circulation [214-217]. Moreover, a large number of primary amines present on the nanoparticle surface allowed for conjugation not only of riboflavin but also a variety of ligands, including the cytotoxic agent methotrexate, or fluorescein as a fluorescent marker

[218]. The investigators confirmed that such conjugates could undergo a specific cellular association in cervical carcinoma HeLa cells, with a subsequent potent inhibition of tumour cell growth, by using a methotrexate conjugate. Alternately, fluorescein could be delivered as an imaging agent for diagnostic purposes.

CONCLUDING REMARKS

In this Chapter, an overview was provided of the breadth of chemical reactions that are catalysed by cofactor-containing enzymes. Generally, cofactors are exploited by oxidoreductases, the class of enzymes that perform the redox reactions that are especially valuable, in selective fashion, for industrial processes because they are not easily accessible by conventional chemical approaches. Yet, in order to extend the capabilities of cofactor-containing enzymes, a series of schemes have been undertaken either to redesign natural cofactors, or to design new cofactor types. Clearly, such engineering efforts will benefit from the previously developed methods that have been used to engineer protein scaffolds, and to accept and tightly bind modified or new ligands. However, the main benefit of this field of enzyme engineering – which combines protein engineering and cofactor redesign – is that it will inevitably lead to the generation of totally new biocatalysts.

REFERENCES

[1] H.B. White, The pyridine nucleotide coenzymes. in: J. Everse, K. Anderson, K. Yu (Eds.), Academic Press, New York, 1982, pp. 1-17.

[2] K. Kruger, P.J. Grabowski, A.J. Zaug, J. Sands, D.E. Gottschling, T.R. Cech, Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena, Cell. 31 (1982) 147-157.

[3] C. Guerrier-Takada, K. Gardiner, T. Marsh, N. Pace, S. Altman, The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme, Cell. 35 (1983) 849-857.

[4] F.H. Crick, The origin of the genetic code, J. Mol. Biol. 38 (1968) 367-379.

[5] L.E. Orgel, Evolution of the genetic apparatus, J. Mol. Biol. 38 (1968) 381-393.

[6] R.F. Gesteland, T.R. Cech, The RNA World: The Nature of Modern RNA Suggests a Prebiotic RNA World, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2006, 768 p.

[7] W.C. Winkler, S. Cohen-Chalamish, R.R. Breaker, An mRNA structure that controls gene expression by binding FMN, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 15908-15913.

[8] W. Winkler, A. Nahvi, R.R. Breaker, Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression, Nature. 419 (2002) 952-956.

[9] W.C. Winkler, A. Nahvi, N. Sudarsan, J.E. Barrick, R.R. Breaker, An mRNA structure that controls gene expression by binding S-adenosylmethionine, Nat. Struct. Biol. 10 (2003) 701-707.

[10] W.C. Winkler, A. Nahvi, A. Roth, J.A. Collins, R.R. Breaker, Control of gene expression by a natural metabolite-responsive ribozyme, Nature. 428 (2004) 281-286.

[11] M. Mandal, B. Boese, J.E. Barrick, W.C. Winkler, R.R. Breaker, Riboswitches control fundamental biochemical pathways in *Bacillus subtilis* and other bacteria, Cell. 113 (2003) 577-586.

[12] M. Mandal, M. Lee, J.E. Barrick, et al., A glycine-dependent riboswitch that uses cooperative binding to control gene expression, Science. 306 (2004) 275-279.

[13] M. Mandal, R.R. Breaker, Adenine riboswitches and gene activation by disruption of a transcription terminator, Nat. Struct. Mol. Biol. 11 (2004) 29-35.

[14] K.A. Corbino, J.E. Barrick, J. Lim, et al., Evidence for a second class of S-adenosylmethionine riboswitches and other regulatory RNA motifs in alpha-proteobacteria, Genome Biol. 6 (2005) R70.

[15] R.T. Fuchs, F.J. Grundy, T.M. Henkin, The S(MK) box is a new SAM-binding RNA for translational regulation of SAM synthetase, Nat. Struct. Mol. Biol. 13 (2006) 226-233.

[16] J.N. Kim, A. Roth, R.R. Breaker, Guanine riboswitch variants from *Mesoplasma florum* selectively recognize 2'-deoxyguanosine, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 16092-16097.

[17] A. Roth, W.C. Winkler, E.E. Regulski, et al., A riboswitch selective for the queuosine precursor preQ1 contains an unusually small aptamer domain, Nat. Struct. Mol. Biol. 14 (2007) 308-317.

[18] W.C. Winkler, R.R. Breaker, Regulation of bacterial gene expression by riboswitches, Annu. Rev. Microbiol. 59 (2005) 487-517.

[19] H.B. White 3rd, Coenzymes as fossils of an earlier metabolic state, J. Mol. Evol. 7 (1976) 101-104.

[20] T.A. Steitz, J.A. Steitz, A general two-metal-ion mechanism for catalytic RNA, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 6498-6502.

[21] R. Hanna, J.A. Doudna, Metal ions in ribozyme folding and catalysis, Curr. Opin. Chem. Biol. 4 (2000) 166-170.

[22] E. Szathmáry, The origin of the genetic code: amino acids as cofactors in an RNA world, Trends Genet. 15 (1999) 223-229.

[23] T. Sekimoto, T. Matsuyama, T. Fukui, K. Tanizawa, Evidence for lysine 80 as general base catalyst of leucine dehydrogenase, J. Biol. Chem. 268 (1993) 27039-27045.

[24] J. Maynard Smith, E. Szathmáry, The Major Transitions in Evolution, W.H. Freeman Spektrum, Oxford etc., 1995

[25] E. Szathmary, J.M. Smith, The major evolutionary transitions, Nature. 374 (1995) 227-232.

[26] A.D. McNaught, A. Wilkinson, IUPAC. Compendium of Chemical Terminology, 2nd ed., Blackwell Scientific Publications, Oxford, 1997.

[27] J.D. Fischer, G.L. Holliday, S.A. Rahman, J.M. Thornton, The structures and physicochemical properties of organic cofactors in biocatalysis, J. Mol. Biol. 403 (2010) 803-824.

[28] C. Andreini, I. Bertini, G. Cavallaro, G.L. Holliday, J.M. Thornton, Metal ions in biological catalysis: from enzyme databases to general principles, J. Biol. Inorg. Chem. 13 (2008) 1205-1218.

[29] http://www.ebi.ac.uk/thornton-srv/databases/Metal-MACiE/home.html,.

[30] J.D. Fischer, G.L. Holliday, J.M. Thornton, The CoFactor database: organic cofactors in enzyme catalysis, Bioinformatics. 26 (2010) 2496-2497.

[31] http://www.ebi.ac.uk/thornton-srv/databases/CoFactor/,.

[32] R.A. Schmitz, S.P. Albracht, R.K. Thauer, A molybdenum and a tungsten isoenzyme of formylmethanofuran dehydrogenase in the thermophilic archaeon *Methanobacterium wolfei*, Eur. J. Biochem. 209 (1992) 1013-1018.

[33] R.H. Holm, P. Kennepohl, E.I. Solomon, Structural and Functional Aspects of Metal Sites in Biology, Chem. Rev. 96 (1996) 2239-2314.

[34] E.I. Solomon, T.C. Brunold, M.I. Davis, et al., Geometric and electronic structure/function correlations in non-heme iron enzymes, Chem. Rev. 100 (2000) 235-350.

[35] M.L. Neidig, E.I. Solomon, Structure-function correlations in oxygen activating non-heme iron enzymes, Chem. Commun. (Camb). (47) (2005) 5843-5863.

[36] I.M. Klotz, D.M. Kurtz Jr., Binuclear oxygen carriers: hemerythrin, Acc. Chem. Res. 17 (1984) 16-22.

[37] J. Stubbe, Ribonucleotide reductases: amazing and confusing, J. Biol. Chem. 265 (1990) 5329-5332.

[38] P. Nordlund, B.M. Sjoberg, H. Eklund, Three-dimensional structure of the free radical protein of ribonucleotide reductase, Nature. 345 (1990) 593-598.

[39] P. Nordlund, H. Eklund, Structure and function of the *Escherichia coli* ribonucleotide reductase protein R2, J. Mol. Biol. 232 (1993) 123-164.

[40] U. Uhlin, H. Eklund, Structure of ribonucleotide reductase protein R1, Nature. 370 (1994) 533-539.

[41] M.P. Woodland, H. Dalton, Purification and characterization of component A of the methane monooxygenase from *Methylococcus capsulatus* (Bath), J. Biol. Chem. 259 (1984) 53-59.

[42] B.G. Fox, J.D. Lipscomb, Purification of a high specific activity methane monooxygenase hydroxylase component from a type II methanotroph, Biochem. Biophys. Res. Commun. 154 (1988) 165-170.

[43] B.G. Fox, W.A. Froland, J.E. Dege, J.D. Lipscomb, Methane monooxygenase from *Methylosinus trichosporium* OB3b. Purification and properties of a three-component system with high specific activity from a type II methanotroph, J. Biol. Chem. 264 (1989) 10023-10033.

[44] B.G. Fox, J. Shanklin, C. Somerville, E. Munck, Stearoyl-acyl carrier protein delta 9 desaturase from *Ricinus communis* is a diiron-oxo protein, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 2486-2490.

[45] D.A. Kopp, S.J. Lippard, Soluble methane monooxygenase: activation of dioxygen and methane, Curr. Opin. Chem. Biol. 6 (2002) 568-576.

[46] E.G. Kovaleva, M.B. Neibergall, S. Chakrabarty, J.D. Lipscomb, Finding intermediates in the O_2 activation pathways of non-heme iron oxygenases, Acc. Chem. Res. 40 (2007) 475-483.

[47] C. Huber, W. Eisenreich, S. Hecht, G. Wachtershauser, A possible primordial peptide cycle, Science. 301 (2003) 938-940.

[48] G. Wachtershauser, On the chemistry and evolution of the pioneer organism, Chem. Biodivers. 4 (2007) 584-602.

[49] H. Beinert, R.H. Holm, E. Munck, Iron-sulfur clusters: nature's modular, multipurpose structures, Science. 277 (1997) 653-659.

[50] C.M. Gorst, Y.H. Yeh, Q. Teng, et al., ^{1}H NMR investigation of the paramagnetic cluster environment in *Pyrococcus furiosus* three-iron ferredoxin: sequence-specific assignment of ligated cysteines independent of tertiary structure, Biochemistry. 34 (1995) 600-610.

[51] L.M. Hunsicker-Wang, A. Heine, Y. Chen, et al., High-resolution structure of the soluble, respiratory-type Rieske protein from *Thermus thermophilus*: analysis and comparison, Biochemistry. 42 (2003) 7303-7317.

[52] J. Meyer, Iron-sulfur protein folds, iron-sulfur chemistry, and evolution, J. Biol. Inorg. Chem. 13 (2008) 157-170.

[53] C.H. Snyder, T. Merbitz-Zahradnik, T.A. Link, B.L. Trumpower, Role of the Rieske iron-sulfur protein midpoint potential in the protonmotive Q-cycle mechanism of the cytochrome bc1 complex, J. Bioenerg. Biomembr. 31 (1999) 235-242.

[54] T. Glaser, B. Hedman, K.O. Hodgson, E.I. Solomon, Ligand K-edge X-ray absorption spectroscopy: a direct probe of ligand-metal covalency, Acc. Chem. Res. 33 (2000) 859-868.

[55] D.C. Johnson, D.R. Dean, A.D. Smith, M.K. Johnson, Structure, function, and formation of biological iron-sulfur clusters, Annu. Rev. Biochem. 74 (2005) 247-281.

[56] G.M. Ullmann, L. Noodleman, D.A. Case, Density functional calculation of p K(a) values and redox potentials in the bovine Rieske iron-sulfur protein, J. Biol. Inorg. Chem. 7 (2002) 632-639.

[57] R.B. King, Encyclopedia of Inorganic Chemistry, (1994).

[58] J.W. Peters, M.H. Stowell, S.M. Soltis, M.G. Finnegan, M.K. Johnson, D.C. Rees, Redox-dependent structural changes in the nitrogenase P-cluster, Biochemistry. 36 (1997) 1181-1187.

[59] A. Harden, W.J. Young, The Alcoholic Ferment of Yeast-Juice. Part II.–The Conferment of Yeast-Juice, Proceedings of the Royal Society B. 78 (1906) 369-375.

[60] O. Warburg, W. Christian, Pyridine, the hydrogen-transferring component of the fermentation enzymes. (Pyridine nucleotide), Biochem. Z. 287 (1936) 291-328.

[61] P. Walter, N.O. Kaplan, Substituted Nicotinamide Analogues of Nicotinamide Adenine Dinucleotide, J. Biol. Chem. 238 (1963) 2823-2830.

[62] C.P. Fawcett, N.O. Kaplan, Preparation and properties of some nicotinamide adenine dinucleotide analogues with pentose and purine modifications, J. Biol. Chem. 237 (1962) 1709-1715.

[63] B.M. Anderson, N.O. Kaplan, Enzymatic studies with analogues of diphosphopyridine nucleotide, J. Biol. Chem. 234 (1959) 1226-1232.

[64] B.M. Anderson, C.J. Ciotti, N.O. Kaplan, Chemical properties of 3-substituted pyridine analogues of diphosphopyridine nucleotide, J. Biol. Chem. 234 (1959) 1219-1225.

[65] M.M. Ciotti, N.O. Kaplan, Chemistry and properties of the 3-acetylpyridine analogue of diphosphopyridine nucleotide, J. Biol. Chem. 221 (1956) 823-832.

[66] M.M. Ciotti, N.O. Kaplan, F.E. Stolzenbach, Reaction of pyridine nucleotide analogues with dehydrogenases, J. Biol. Chem. 221 (1956) 833-844.

[67] B.M. Anderson, C.D. Anderson, Preparation and characterization of the NAD vinylogue, 3-pyridylacryloamide adenine dinucleotide, Anal. Biochem. 195 (1991) 57-62.

[68] B.M. Anderson, C.D. Anderson, J.K. Lee, A.M. Stein, The Thionicotinamide Analogs of DPN and TPN. II. Enzyme Studies, Biochemistry. 2 (1963) 1017-1022.

[69] D. Eby, M.E. Kirtley, Interaction of nicotinamide-adenine dinucleotide and its analogs with glyceraldehyde 3-phosphate dehydrogenase, Biochemistry. 10 (1971) 2677-2682.

[70] J.P. Samama, A.D. Wrixon, J.F. Biellmann, 5-Methylnicotinamide-adenine dinucleotide. Kinetic investigation with major and minor isoenzymes of liver alcohol dehydrogenase and structural determination of its binary complex with alcohol dehydrogenase, Eur. J. Biochem. 118 (1981) 479-486.

[71] M.A. Abdallah, J.F. Biellmann, J.P. Samama, A.D. Wrixon, Preparation and properties of 3-halopyridine--adenine dinucleotides, NAD^+ analogues and model compounds, Eur. J. Biochem. 64 (1976) 351-360.

[72] E.J. Freyne, E.L. Esmans, T.O. Van Osselaer, J.A. Lepoivre, F.C. Alderweireldt, Studies on NAD^+ analogs. Part II. Purification by HPLC and enzymic activity of 3-Ac-5-alkylPyAD^+, Journal of Carbohydrates, Nucleosides, Nucleotides. 8 (1981) 537-545.

[73] S. Tono-Oka, Y. Sasahara, A. Sasaki, H. Shirahama, T. Matsumoto, S. Kakimoto, Enzymic synthesis of new pyridine nucleosides. Clitidine and its amide derivative, Bull. Chem. Soc. Jap. 54 (1981) 212-216.

[74] A. Baici, P.L. Luisi, O. Attanasi, The kinetic mechanism of horse liver alcohol dehydrogenase with thio-NAD as coenzyme, J. Mol. Catal. 1 (1976) 223-244.

[75] N.A. Beijer, H.M. Buck, L.A. Sluyterman, E.M. Meijer, NAD^+ and NAD^+ analogues in horse liver alcohol dehydrogenase. Relationship between reactivity and conformation simulated with molecular mechanics, Biochim. Biophys. Acta. 1039 (1990) 227-233.

[76] Y.Q. Shen, S.Y. Song, Z.J. Lin, Structures of D-glyceraldehyde-3-phosphate dehydrogenase complexed with coenzyme analogues, Acta Crystallogr. D Biol. Crystallogr. 58 (2002) 1287-1297.

[77] R.J. Kazlauskas, Changing coenzymes improves oxidations catalyzed by alcohol dehydrogenase, J. Org. Chem. 53 (1988) 4633-4635.

[78] C. Zheng, R.S. Phillips, Effect of coenzyme analogs on enantioselectivity of alcohol dehydrogenase. [Erratum to document cited in CA117(7):69418u], J. Chem. Soc. , Perkin I. 18 (1992) 1083-1084.

[79] C.P. Salerno, D. Magde, A.P. Patron, Enzymatic synthesis of caged NADP cofactors: aqueous NADP photorelease and optical properties, J. Org. Chem. 65 (2000) 3971-3981.

[80] C.P. Salerno, M. Resat, D. Magde, J. Kraut, Synthesis of Caged NAD (P) Coenzymes: Photorelease of NADP, J. Am. Chem. Soc. 119 (1997) 3403-3404.

[81] G. Mayer, A. Heckel, Biologically active molecules with a "light switch", Angewandte Chemie International Edition. 45 (2006) 4900-4921.

[82] J.C. Lambry, E. Beaumont, B. Tarus, M. Blanchard-Desce, A. Slama-Schwok, Selective probing of a NADPH site controlled light-induced enzymatic catalysis, Journal of Molecular Recognition. 23 (2010) 379-388.

[83] W. Liu, M. Gaines, R. Bao, J. Clifford, Y. Li, Preparation and Evaluation of Arylazide-Substituted Pyridine Adenine Dinucleotides for Photoaffinity Labeling Experiments, Photochem. Photobiol. 63 (1996) 793-799.

[84] R. Stewart, D.J. Norris, The pyridinium-dihydropyridine system. Part 2. Substituent effects on the oxidation of 1,4-dihydropyridines by flavins, J. Chem. Soc. , Perkin II. 3 (1978) 246-249.

[85] D.J. Norris, R. Stewart, The pyridinium-dihydropyridine system. I. Synthesis of a series of substituted pyridinium ions and their 1,4-dihydro reduction products and a determination of their stabilities in aqueous buffers, Can. J. Chem. 55 (1977) 1687-1695.

[86] J.T. Slama, A.M. Simmons, Carbanicotinamide adenine dinucleotide: synthesis and enzymological properties of a carbocyclic analogue of oxidized nicotinamide adenine dinucleotide, Biochemistry. 27 (1988) 183-193.

[87] S. Sicsic, P. Durand, S. Langrene, F. Le Goffic, Activity of NMN^+, nicotinamide ribose and analogs in alcohol oxidation promoted by horse-liver alcohol dehydrogenase. Improvement of this activity and structural requirements of the pyridine nucleotide part of the NAD^+ coenzyme, Eur. J. Biochem. 155 (1986) 403-407.

[88] S. Sicsic, P. Durand, S. Langrene, F. le Goffic, A new approach for using cofactor dependent enzymes: example of alcohol dehydrogenase, FEBS Lett. 176 (1984) 321-330.

[89] H.K. Chenault, G.M. Whitesides, Regeneration of nicotinamide cofactors for use in organic synthesis, Appl. Biochem. Biotechnol. 14 (1987) 147-197.

[90] R. Wichmann, C. Wandrey, A.F. Bueckmann, M.R. Kula, Continuous enzymic transformation in an enzyme membrane reactor with simultaneous NAD(H) regeneration, Biotechnol. Bioeng. 23 (1981) 2789-2802.

[91] N. Katayama, K. Hayakawa, I. Urabe, H. Okada, Kinetic properties of N6-(2-carboxyethyl)-NAD(H) and poly(ethylene glycol)-bound NAD(H) for alcohol, lactate, malate and glyceraldehyde-3-phosphate dehydrogenase from different organisms, Enzyme Microb. Technol. 6 (1984) 538-542.

[92] Y. Yamazaki, H. Maeda, A. Satoh, K. Hiromi, A kinetic study on the binding of monomeric and polymeric derivatives of NAD+ to yeast alcohol dehydrogenase, J. Biochem. 95 (1984) 109-115.

[93] W. Leuchtenberger, M. Karrenbauer, U. Ploecker, Scale-up of an enzyme membrane reactor process for the manufacture of L-enantiomeric compounds, Ann. N.Y. Acad. Sci. 434 (1984) 78-86.

[94] T. Ohshima, C. Wandrey, M.R. Kula, K. Soda, Improvement for L-leucine production in a continuously operated enzyme membrane reactor, Biotechnol. Bioeng. 27 (1985) 1616-1618.

[95] S. Riva, G. Carrea, F.M. Veronese, A.F. Bückmann, Effect of coupling site and nature of the polymer on the coenzymatic properties of water-soluble macromolecular NAD derivatives with selected dehydrogenase enzymes, Enzyme Microb. Technol. 8 (1986) 556-560.

[96] M. Stengelin, R.N. Patel, Phenylalanine Dehydrogenase Catalyzed Reductive Amination of 6-(1', 3'-Dioxolan-2'-YL)-2-Keto-Hexanoic Acid to 6-(1', 3'-Dioxolan-2'-YL)-2S-Aminohexanoic Acid with Nadh Regeneration and Enzyme and Cofactor Retention, Biocatal. Biotransform. 18 (2000) 373-400.

[97] M. Ikemi, Y. Ishimatsu, The membrane bioreactor with coenzyme recycling system, J. Biotechnol. 14 (1990) 211-220.

[98] K.D. Kulbe, U. Schwab , M. Howaldt, Conjugated NAD (H)-dependent Dehydrogenases for the Continuous Production of Mannitol and Gluconic Acid from Glucose-Fructose Mixtures in a Membrane Reactor, Ann. N.Y. Acad. Sci. 501 (1987) 216-223.

[99] E. Steckhan, S. Herrmann, R. Ruppert, J. Thömmes, C. Wandrey, Continuous Generation of NADH from NAD^+ and Formate Using a Homogeneous Catalyst with Enhanced Molecular Weight in a Membrane Reactor, Angewandte Chemie International Edition in English. 29 (1990) 388-390.

[100] S.A.M. Vanhommerig, L.A. Sluyterman, E.M. Meijer, Kinetic and modelling studies of NAD and poly (ethylene glycol)-bound NAD in horse liver alcohol dehydrogenase, Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology. 1295 (1996) 125-138.

[101] R.J. Ansell, D.A.P. Small, C.R. Lowe, The interactions of artificial coenzymes with alcohol dehydrogenase and other NAD (P)(H) dependent enzymes, J Molec Catal B. 6 (1999) 111-123.

[102] R.J. Ansell, D.A.P. Small, C.R. Lowe, Synthesis and properties of new coenzyme mimics based on the artificial coenzyme CL4, Journal of Molecular Recognition. 12 (1999) 45-56.

[103] R. Ansell, C. Lowe, Artificial redox coenzymes: biomimetic analogues of NAD, Appl. Microbiol. Biotechnol. 51 (1999) 703-710.

[104] S.B. McLoughlin, C.R. Lowe, An enzymatically active artificial redox coenzyme based on a synthetic dye template, Enzyme Microb. Technol. 20 (1997) 2-11.

[105] S. Dilmaghanian, C. Vivian Stead, R.J. Ansell, C.R. Lowe, Synthesis and properties of a naphthalene-containing artificial redox coenzyme, Enzyme Microb. Technol. 20 (1997) 165-173.

[106] R.J. Ansell, D.A.P. Small, C.R. Lowe, Characterisation of the artificial coenzyme CL4, J. Molec Catal B. 3 (1997) 239-252.

[107] R.J. Ansell, S. Dilmaghanian, C.V. Stead, C.R. Lowe, Synthesis and properties of new coenzyme mimics based on the artificial coenzyme Blue N-3, Enzyme Microb. Technol. 21 (1997) 327-334.

[108] S.J. Burton, C. Vivian Stead, R.J. Ansell, C.R. Lowe, An artificial redox coenzyme based on a triazine dye template, Enzyme Microb. Technol. 18 (1996) 570-580.

[109] J.D. Ryan, R.H. Fish, D.S. Clark, Engineering Cytochrome P450 Enzymes for Improved Activity towards Biomimetic 1, 4-NADH Cofactors, ChemBioChem. 9 (2008) 2579-2582.

[110] J. Lutz, F. Hollmann, Bioorganometallic chemistry: biocatalytic oxidation reactions with biomimetic NAD^+/NADH co-factors and [Cp* Rh (bpy) H] for selective organic synthesis, J Organomet Chem 689 (2004) 4783-4790.

[111] H.C. Lo, R.H. Fish, Biomimetic NAD^+ Models for Tandem Cofactor Regeneration, Horse Liver Alcohol Dehydrogenase Recognition of 1, 4-NADH Derivatives, and Chiral Synthesis, Angew. Chem. Int. Ed. 41 (2002) 478-481.

[112] A. Alfieri, E. Malito, R. Orru, M.W. Fraaije, A. Mattevi, Revealing the moonlighting role of NADP in the structure of a flavin-containing monooxygenase, Proceedings of the National Academy of Sciences. PNAS. 105 (2008) 6572-6577.

[113] D. Ji, L. Wang, S. Hou, et al., Creation of Bioorthogonal Redox Systems Depending on Nicotinamide Flucytosine Dinucleotide, J. Am. Chem. Soc. 133 (2011) 20857-20862.

[114] S. Abe, T. Ueno, Y. Watanabe, Artificial metalloproteins exploiting vacant space: preparation, structures, and functions, Bio-inspired Catalysts. (2009) 25-43.

[115] T.R. Ward, Artificial Metalloenzymes Based on the Biotin–Avidin Technology: Enantioselective Catalysis and Beyond, Acc. Chem. Res. 44 (2011) 47-57.

[116] M.R. Ringenberg, T.R. Ward, Merging the best of two worlds: artificial metalloenzymes for enantioselective catalysis, Chem. Commun. (2011).

[117] J. Steinreiber, T.R. Ward, Artificial metalloenzymes as selective catalysts in aqueous media, Coord. Chem. Rev. 252 (2008) 751-766.

[118] F. Rosati, G. Roelfes, Artificial Metalloenzymes, ChemCatChem. 2 (2010) 916-927.

[119] P.J. Deuss, R. den Heeten, W. Laan, P.C.J. Kamer, Bioinspired Catalyst Design and Artificial Metalloenzymes, Chem. Eur. J.17 (2011) 4680-4698.

[120] G.A. Ordway, D.J. Garry, Myoglobin: an essential hemoprotein in striated muscle, J. Exp. Biol. 207 (2004) 3441.

[121] T. Takano, Structure of myoglobin refined at 2.0 resolution: II. Structure of deoxymyoglobin from sperm whale, J. Mol. Biol. 110 (1977) 569-584.

[122] T. Hayashi, T. Takimura, H. Ogoshi, Photoinduced singlet electron transfer in a complex formed from zinc myoglobin and methyl viologen: Artificial recognition by a chemically modified porphyrin, J. Am. Chem. Soc. 117 (1995) 11606-11607.

[123] T. Hayashi, H. Ogoshi, Molecular modelling of electron transfer systems by noncovalently linked porphyrin–acceptor pairing, Chem. Soc. Rev. 26 (1997) 355-364.

[124] T. Hayashi, A. Tomokuni, T. Mizutani, Y. Hisaeda, H. Ogoshi, Interfacial recognition between reconstituted myoglobin having charged binding domain and electron acceptor *via* electrostatic interaction, Chem. Lett. (1998) 1229-1230.

[125] T. Hayashi, Y. Hitomi, T. Takimura, et al., New approach to the construction of an artificial hemoprotein complex, Coord. Chem. Rev. 190 (1999) 961-974.

[126] T. Hayashi, Y. Hisaeda, New functionalization of myoglobin by chemical modification of heme-propionates, Acc. Chem. Res. 35 (2002) 35-43.

[127] T. Hayashi, Y. Hitomi, T. Ando, et al., Peroxidase activity of myoglobin is enhanced by chemical mutation of heme-propionates, J. Am. Chem. Soc. 121 (1999) 7747-7750.

[128] H. Sato, T. Hayashi, T. Ando, Y. Hisaeda, T. Ueno, Y. Watanabe, Hybridization of modified-heme reconstitution and distal histidine mutation to functionalize sperm whale myoglobin, J. Am. Chem. Soc. 126 (2004) 436-437.

[129] T. Hayashi, T. Matsuda, Y. Hisaeda, Enhancement of peroxygenase activity of horse heart myoglobin by modification of heme-propionate side chains, Chem. Lett. 32 (2003) 496-497.

[130] T. Hayashi, H. Dejima, T. Matsuo, H. Sato, a. Dai Murata, Y. Hisaeda, Blue myoglobin reconstituted with an iron porphycene shows extremely high oxygen affinity, J. Am. Chem. Soc. 124 (2002) 11226-11227.

[131] T. Katsuki, Catalytic asymmetric oxidations using optically active (salen) manganese (III) complexes as catalysts, Coord. Chem. Rev. 140 (1995) 189-214.

[132] M. Ohashi, T. Koshiyama, T. Ueno, M. Yanase, H. Fujii, Y. Watanabe, Preparation of artificial metalloenzymes by insertion of chromium (III) Schiff base complexes into apomyoglobin mutants, Angewandte Chemie International Edition. 42 (2003) 1005-1008.

[133] J.L. Zhang, D.K. Garner, L. Liang, Q. Chen, Y. Lu, Protein scaffold of a designed metalloenzyme enhances the chemoselectivity in sulfoxidation of thioanisole, Chem.Commun. (2008) 1665-1667.

[134] T. Ueno, M. Ohashi, M. Kono, et al., Crystal structures of artificial metalloproteins: tight binding of FeIII (Schiff-base) by mutation of Ala71 to Gly in apo-myoglobin, Inorg. Chem. 43 (2004) 2852-2858.

[135] T. Ueno, T. Koshiyama, M. Ohashi, et al., Coordinated design of cofactor and active site structures in development of new protein catalysts, J. Am. Chem. Soc. 127 (2005) 6556-6562.

[136] J.L. Zhang, D.K. Garner, L. Liang, D.A. Barrios, Y. Lu, Noncovalent Modulation of pH-Dependent Reactivity of a Mn–Salen Cofactor in Myoglobin with Hydrogen Peroxide, Chem. Eur. J. 15 (2009) 7481-7489.

[137] J.R. Carey, S.K. Ma, T.D. Pfister, et al., A site-selective dual anchoring strategy for artificial metalloprotein design, J. Am. Chem. Soc. 126 (2004) 10812-10813.

[138] D.K. Garner, L. Liang, D.A. Barrios, J.L. Zhang, Y. Lu, The Important Role of Covalent Anchor Positions in Tuning Catalytic Properties of a Rationally Designed MnSalen-Containing Metalloenzyme, ACS catalysis. (2011) 1083–1089

[139] N. Yokoi, T. Ueno, M. Unno, T. Matsui, M. Ikeda-Saito, Y. Watanabe, Ligand design for the improvement of stability of metal complex·protein hybrids, Chem. Commun. 47 (2008) 229-231.

[140] S. Abe, T. Ueno, P.A.N. Reddy, et al., Design and structure analysis of artificial metalloproteins: Selective coordination of his64 to copper complexes with square-planar structure in the apo-myoglobin scaffold, Inorg. Chem. 46 (2007) 5137-5139.

[141] Y. Satake, S. Abe, S. Okazaki, et al., Incorporation of a Phebox Rhodium Complex into apo-Myoglobin Affords a Stable Organometallic Protein Showing Unprecedented Arrangement of the Complex in the Cavity, Organometallics. 26 (2007) 4904-4908.

[142] E. Zahavy, I. Willner, Photoinduced Electron Transfer in Eosin-Modified Co (II)-Protoporphyrin IX Reconstituted Myoglobin and α-or β-Hemoglobin Subunits: Photocatalytic Transformations by the Reconstituted Photoenzymes1, J. Am. Chem. Soc. 118 (1996) 12499-12514.

[143] E. Zahavy, I. Willner, Application of Eosin-Modified Reconstituted Co (II)-Myoglobin as Semisynthetic Photoenzyme for Cyclic Photosynthesis, J. Am. Chem. Soc. 117 (1995) 10581-10582.

[144] I. Willner, E. Zahavy, V. Heleg-Shabtai, Eosin-Modified Reconstituted Co (II) Protoporphyrin IX Myoglobin: A Semisynthetic Photoenzyme for H2 Evolution and Hydrogenation, J. Am. Chem. Soc. 117 (1995) 542-543.

[145] S. Curry, H. Mandelkow, P. Brick, N. Franks, Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites, Nature Struct. Biol. (1998) 827-835.

[146] S.V. Dzyuba, A.M. Klibanov, Stereoselective oxidations and reductions catalyzed by nonredox proteins, Tetrahedron: Asymmetry. 15 (2004) 2771-2777.

[147] T. Sagawa, H. Ishida, K. Urabe, K. Yoshinaga, K. Ohkubo, Enzymatic tryptophan 2, 3-dioxygenase-like activity of a manganese porphyrin bound to bovine serum albumin modified with poly (ethylene glycol), J. Chem. Soc., Perkin Trans. 2. (1993) 1-5.

[148] T. Sagawa, H. Ishida, K. Urabe, K. Ohkubo, Stereoselective dioxygenolysis of a tryptophan derivative catalyzed by a manganese porphyrin bound to bovine serum albumin, Chem. Lett. 20 (1991) 2083-2086.

[149] A. Mahammed, Z. Gross, Albumin-conjugated corrole metal complexes: extremely simple yet very efficient biomimetic oxidation systems, J. Am. Chem. Soc. 127 (2005) 2883-2887.

[150] A. Mahammed, H.B. Gray, J.J. Weaver, K. Sorasaenee, Z. Gross, Amphiphilic corroles bind tightly to human serum albumin, Bioconjug. Chem. 15 (2004) 738-746.

[151] P. Rousselot-Pailley, C. Bochot, C. Marchi-Delapierre, et al., The protein environment drives selectivity for sulfide oxidation by an artificial metalloenzyme, ChemBioChem. 10 (2009) 545-552.

[152] M. Reetz, N. Jiao, Serum Albumins Host Copper-Catalyzed Diels-Alder Reactions, Angew. Chem. Int. Ed. 45 (2006) 2416-2419.

[153] S. Ghisla, C. Thorpe, Acyl-CoA dehydrogenases. A mechanistic overview, Eur. J. Biochem. 271 (2004) 494-508.

[154] P.F. Fitzpatrick, Oxidation of amines by flavoproteins, Arch. Biochem. Biophys. 493 (2010) 13-25.

[155] D. Fass, The Erv family of sulfhydryl oxidases, Biochim. Biophys. Acta. 1783 (2008) 557-566.

[156] A. Vrielink, S. Ghisla, Cholesterol oxidase: biochemistry and structural features, FEBS J. 276 (2009) 6826-6843.

[157] H.R. Ellis, The FMN-dependent two-component monooxygenase systems, Arch. Biochem. Biophys. 497 (2010) 1-12.

[158] B.A. Palfey, C.A. McDonald, Control of catalysis in flavin-dependent monooxygenases, Arch. Biochem. Biophys. 493 (2010) 26-36.

[159] W.J. van Berkel, N.M. Kamerbeek, M.W. Fraaije, Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts, J. Biotechnol. 124 (2006) 670-689.

[160] J.L. Anderson, S.K. Chapman, Molecular mechanisms of enzyme-catalysed halogenation, Mol. Biosyst. 2 (2006) 350-357.

[161] L.C. Blasiak, C.L. Drennan, Structural perspective on enzymatic halogenation, Acc. Chem. Res. 42 (2009) 147-155.

[162] K.H. van Pee, C. Dong, S. Flecks, J. Naismith, E.P. Patallo, T. Wage, Biological halogenation has moved far beyond haloperoxidases, Adv. Appl. Microbiol. 59 (2006) 127-157.

[163] A. Argyrou, J.S. Blanchard, Flavoprotein disulfide reductases: advances in chemistry and function, Prog. Nucleic Acid Res. Mol. Biol. 78 (2004) 89-142.

[164] H.S. Toogood, J.M. Gardiner, N.S. Scrutton, Biocatalytic reductions and chemical versatility of the old yellow enzyme family of flavoprotein oxidoreductases, ChemCatChem. 2 (2010) 892-914.

[165] E. Demarsy , C. Fankhauser, Higher plants use LOV to perceive blue light, Curr Opin Plant Biol. 12 (2009) 69-74.

[166] M. Gomelsky, G. Klug, BLUF: a novel FAD-binding domain involved in sensory transduction in microorganisms, Trends Biochem. Sci. 27 (2002) 497-500.

[167] I.H. Kavakli, A. Sancar, Circadian photoreception in humans and mice, Mol. Interv. 2 (2002) 484-492.

[168] C. Lin, T. Todo, The cryptochromes, Genome Biol. 6 (2005) 220-220.9.

[169] A. Losi, W. Gartner, Old chromophores, new photoactivation paradigms, trendy applications: flavins in blue light-sensing photoreceptors, Photochem. Photobiol. 87 (2011) 491-510.

[170] N. Ozturk, S.H. Song, S. Ozgur, et al., Structure and function of animal cryptochromes, Cold Spring Harb. Symp. Quant. Biol. 72 (2007) 119-131.

[171] S. Braatsch, M. Gomelsky, S. Kuphal, G. Klug, A single flavoprotein, AppA, integrates both redox and light signals in *Rhodobacter sphaeroides*, Mol. Microbiol. 45 (2002) 827-836.

[172] P. Macheroux, S. Hill, S. Austin, et al., Electron donation to the flavoprotein NifL, a redox-sensing transcriptional regulator, Biochem. J. 332 (Pt 2) (1998) 413-419.

[173] H.A. Krebs, Metabolism of amino-acids: Deamination of amino-acids, Biochem. J. 29 (1935) 1620-1644.

[174] O. Warburg, W. Christian, The yellow enzyme and its functions, Biochem. Z. 266 (1933) 377-411.

[175] H. Theorell, Purification of the active group of the yellow enzyme, Biochem. Z. 275 (1935) 344-346.

[176] S. Ghisla, V. Massey, Mechanisms of flavoprotein-catalyzed reactions, Eur. J. Biochem. 181 (1989) 1-17.

[177] L. Fruk, C.H. Kuo, E. Torres, C.M. Niemeyer, Apoenzyme reconstitution as a chemical tool for structural enzymology and biotechnology, Angew. Chem. Int. Ed Engl. 48 (2009) 1550-1574.

[178] V. Massey, Activation of molecular oxygen by flavins and flavoproteins, J. Biol. Chem. 269 (1994) 22459-22462.

[179] V. Massey, G. Palmer, On the existence of spectrally distinct classes of flavoprotein semiquinones. A new method for the quantitative production of flavoprotein semiquinones, Biochemistry. 5 (1966) 3181-3189.

[180] D.P. Heuts, N.S. Scrutton, W.S. McIntire, M.W. Fraaije, What's in a covalent bond? On the role and formation of covalently bound flavin cofactors, FEBS J. 276 (2009) 3405-3427.

[181] P. Macheroux, B. Kappes, S.E. Ealick, Flavogenomics-a genomic and structural view of flavin-dependent proteins, FEBS J. 278 (2011) 2625-2634.

[182] M. Mewies, W.S. McIntire, N.S. Scrutton, Covalent attachment of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to enzymes: the current state of affairs, Protein Sci. 7 (1998) 7-20.

[183] K. Podzelinska, R. Latimer, A. Bhattacharya, L.C. Vining, D.L. Zechel, Z. Jia, Chloramphenicol biosynthesis: the structure of CmlS, a flavin-dependent halogenase showing a covalent flavin-aspartate bond, J. Mol. Biol. 397 (2010) 316-331.

[184] N.G. Leferink, D.P. Heuts, M.W. Fraaije, W.J. van Berkel, The growing VAO flavoprotein family, Arch. Biochem. Biophys. 474 (2008) 292-301.

[185] H.L. Levine, Y. Nakagawa, E.T. Kaiser, Flavopapain: synthesis and properties of semi-synthetic enzymes, Biochem. Biophys. Res. Commun. 76 (1977) 64-70.

[186] J.T. Slama, C. Radziejewski, S. Oruganti, E.T. Kaiser, Semisynthetic enzymes: characterization of isomeric flavopapains with widely different catalytic efficiencies, J. Am. Chem. Soc. 106 (1984) 6778-6785.

[187] M.A. Wagner, P. Khanna, M.S. Jorns, Structure of the flavocoenzyme of two homologous amine oxidases: monomeric sarcosine oxidase and N-methyltryptophan oxidase, Biochemistry. 38 (1999) 5588-5595.

[188] A. Hassan-Abdallah, R.C. Bruckner, G. Zhao, M.S. Jorns, Biosynthesis of covalently bound flavin: isolation and *in vitro* flavinylation of the monomeric sarcosine oxidase apoprotein, Biochemistry. 44 (2005) 6452-6462.

[189] J. Kim, J.H. Fuller, V. Kuusk, et al., The cytochrome subunit is necessary for covalent FAD attachment to the flavoprotein subunit of p-cresol methylhydroxylase, J. Biol. Chem. 270 (1995) 31202-31209.

[190] R. Brandsch, V. Bichler, B. Krauss, Binding of FAD to 6-hydroxy-D-nicotine oxidase apoenzyme prevents degradation of the holoenzyme, Biochem. J. 258 (1989) 187-192.

[191] R. Brandsch, V. Bichler, Autoflavinylation of apo6-hydroxy-D-nicotine oxidase, J. Biol. Chem. 266 (1991) 19056-19062.

[192] J. Jin, H. Mazon, R.H. van den Heuvel, A.J. Heck, D.B. Janssen, M.W. Fraaije, Covalent flavinylation of vanillyl-alcohol oxidase is an autocatalytic process, FEBS J. 275 (2008) 5191-5200.

[193] E.W. van Hellemond, H. Mazon, A.J. Heck, et al., ADP competes with FAD binding in putrescine oxidase, J. Biol. Chem. 283 (2008) 28259-28264.

[194] M.M. Kopacz, S. Rovida, E. van Duijn, M.W. Fraaije, A. Mattevi, Structure-based redesign of cofactor binding in putrescine oxidase, Biochemistry. 50 (2011) 4209-4217.

[195] P. Trickey, M.A. Wagner, M.S. Jorns, F.S. Mathews, Monomeric sarcosine oxidase: structure of a covalently flavinylated amine oxidizing enzyme, Structure. 7 (1999) 331-345.

[196] C. Walsh, Flavin Coenzymes: At the Crossroads of Biological Redox Chemistry, Acc. Chem. Res. 13 (1980) 148-155.

[197] I. Efimov, W.S. McIntire, A study of the spectral and redox properties and covalent flavinylation of the flavoprotein component of p-cresol methylhydroxylase reconstituted with FAD analogues, Biochemistry. 43 (2004) 10532-10546.

[198] J.W. Koetter, G.E. Schulz, Crystal structure of 6-hydroxy-D-nicotine oxidase from *Arthrobacter nicotinovorans*, J. Mol. Biol. 352 (2005) 418-428.

[199] W. Weyler, Y.P. Hsu, X.O. Breakefield, Biochemistry and genetics of monoamine oxidase, Pharmacol. Ther. 47 (1990) 391-417.

[200] I. Hirashiki, F. Ogata, A. Ito, Rat monoamine oxidase B expressed in *Escherichia coli* has a covalently-bound FAD, Biochem. Mol. Biol. Int. 37 (1995) 39-44.

[201] G. Lu, T. Unge, J.B. Owera-Atepo, J.C. Shih, J. Ekblom, L. Oreland, Characterization and partial purification of human monoamine oxidase-B expressed in *Escherichia coli*, Protein Expr. Purif. 7 (1996) 315-322.

[202] V. Massey, The chemical and biological versatility of riboflavin, Biochem. Soc. Trans. 28 (2000) 283-296.

[203] C. Kemal, T.C. Bruice, Simple synthesis of a 4a-hydroperoxy adduct of a 1,5-dihydroflavine: preliminary studies of a model for bacterial luciferase, Proc. Natl. Acad. Sci. U. S. A. 73 (1976) 995-999.

[204] C. Smit, M.W. Fraaije, A.J. Minnaard, Reduction of carbon-carbon double bonds using organocatalytically generated diimide, J. Org. Chem. 73 (2008) 9482-9485.

[205] Y. Imada, H. Iida, S. Murahashi, T. Naota, An aerobic, organocatalytic, and chemoselective method for Baeyer-Villiger oxidation, Angew. Chem. Int. Ed Engl. 44 (2005) 1704-1706.

[206] G. de Gonzalo, C. Smit, J. Jin, A.J. Minnaard, M.W. Fraaije, Turning a riboflavin-binding protein into a self-sufficient monooxygenase by cofactor redesign, Chem. Commun. (Camb). 47 (2011) 11050-11052.

[207] P. Pattanaik, Sooryanarayana, P.R. Adiga, S.S. Visweswariah, Refolding of native and recombinant chicken riboflavin carrier (or binding) protein: evidence for the formation of non-native intermediates during the generation of active protein, Eur. J. Biochem. 258 (1998) 411-418.

[208] E. van Bloois, R.T. Winter, D.B. Janssen, M.W. Fraaije, Export of functional *Streptomyces coelicolor* alditol oxidase to the periplasm or cell surface of *Escherichia coli* and its application in whole-cell biocatalysis, Appl. Microbiol. Biotechnol. 83 (2009) 679-687.

[209] A.B. Foraker, C.M. Khantwal, P.W. Swaan, Current perspectives on the cellular uptake and trafficking of riboflavin, Adv. Drug Deliv. Rev. 55 (2003) 1467-1483.

[210] A.A. Karande, L. Sridhar, K.S. Gopinath, P.R. Adiga, Riboflavin carrier protein: a serum and tissue marker for breast carcinoma, Int. J. Cancer. 95 (2001) 277-281.

[211] P.N. Rao, J. Crippin, E. Levine, et al., Elevation of serum riboflavin carrier protein in hepatocellular carcinoma, Hepatol. Res. 35 (2006) 83-87.

[212] L.M. Bareford, M.A. Phelps, A.B. Foraker, P.W. Swaan, Intracellular processing of riboflavin in human breast cancer cells, Mol. Pharm. 5 (2008) 839-848.

[213] T. Johnson, A. Ouhtit, R. Gaur, et al., Biochemical characterization of riboflavin carrier protein (RCP) in prostate cancer, Front. Biosci. 14 (2009) 3634-3640.

[214] D.A. Tomalia, A.M. Naylor, W.A.I. Goddard, Starburst dendrimers: control of size, shape, surface chemistry, topology and flexibility in the conversion of atoms to macroscopic materials, Angew. Chem. Int. Ed Engl. 102 (1990) 119-157.

[215] M.J. Cloninger, Biological applications of dendrimers, Curr. Opin. Chem. Biol. 6 (2002) 742-748.

[216] R. Esfand, D.A. Tomalia, Poly(amidoamine) (PAMAM) dendrimers: from biomimicry to drug delivery and biomedical applications, Drug Discov. Today. 6 (2001) 427-436.

[217] S.H. Medina, M.E. El-Sayed, Dendrimers as carriers for delivery of chemotherapeutic agents, Chem. Rev. 109 (2009) 3141-3157.

[218] T.P. Thomas, S.K. Choi, M. Li, A. Kotlyar, J.R. Baker Jr., Design of riboflavin-presenting PAMAM dendrimers as a new nanoplatform for cancer-targeted delivery, Bioorg Med Chem Lett. 20 (2010) 5191-5194.

CHAPTER 3

STRUCTURE-BASED REDESIGN OF COFACTOR BINDING IN PUTRESCINE OXIDASE

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ABSTRACT

Putrescine oxidase (PuO) from *Rhodococcus erythropolis* is a soluble homodimeric flavoprotein, which oxidizes small aliphatic diamines. In this study, we report the crystal structures and cofactor binding properties of wild-type and mutant enzymes. From a structural viewpoint, PuO closely resembles the sequence-related human monoamine oxidases A and B. This similarity is striking in the flavin-binding site even if PuO does not bind covalently the cofactor, differently from monoamine oxidases. A remarkable conserved feature is the *cis* peptide conformation of the Tyr residue which recognizes the substrate. The structure of PuO in complex with the reaction product reveals that Glu324 is crucial in recognizing the terminal amino group of the diamine substrate and explains the narrow substrate specificity of the enzyme. The structural analysis also provides clues for identification of residues that are responsible for the competitive binding of ADP versus FAD (~50% of wild-type PuO monomers isolated are occupied by ADP instead of FAD). By replacing Pro15, which is part of the dinucleotide-binding domain, enzyme preparations were obtained that are almost 100% in the FAD-bound form. Furthermore, mutants have been designed and prepared that form a covalent 8α-*S*-cysteinyl-FAD linkage. These data provide new insights into the molecular basis for substrate recognition in amine oxidases and demonstrate that engineering of flavoenzymes to introduce covalent linkage with the cofactor is a possible route to develop more stable protein molecules, better suited for biocatalytic purposes.

INTRODUCTION

Flavin cofactors FMN and FAD are essential for the catalytic function of a wide range of proteins. In most cases they are tightly bound to proteins as prosthetic groups by dedicated cofactor-binding domains. Analysis of the available crystal structures of FAD-containing proteins reveals several structural classes [1]. The majority of these FAD-containing proteins contain a distinct domain that binds tightly but in a non-covalent manner the ADP moiety of the FAD molecule. In many cases this is a Rossmann fold dinucleotide-binding domain which can be easily recognized at sequence level by the canonical GxGxxG sequence motif. Another layer of complexity concerning flavin cofactor binding is added by the observation that some flavoproteins contain a covalently bound flavin cofactor. Several types of covalent protein-FAD linkages have been found [2]. The most common covalent bond involves the C8α-methyl moiety of the flavin cofactor and a histidine or cysteine residue. The role of the covalent flavin-protein bond appears to vary from protein to protein. In some flavoproteins the covalent linkage is required for tuning the redox properties of the flavin or for increasing protein stability. Flavin incorporation has been shown to be an auto-catalytic process which is in contrast with other covalently bound cofactors, e.g. heme in cytochrome c, that depend on dedicated enzymes that catalyse covalent cofactor incorporation [3-7].

Putrescine oxidase from *Rhodococcus erythropolis* (PuO) is a recently discovered amine oxidase which contains a non-covalently bound FAD molecule [8]. It shows a high degree of sequence homology with human and fungal monoamine oxidases (MAOs) which all are active on a broad range of aromatic compounds [9,10]. Nevertheless, PuO only accepts a few small aliphatic diamines as substrates, e.g. putrescine and cadaverine. Protomers of this enzyme are predicted to bind one FAD because of the presence of the typical dinucleotide-binding motif (DBM). However, PuO is unique among all reported flavoprotein oxidases in that it is found to contain one FAD molecule per protein dimer [11,12]. Mass spectrometry (MS) analysis has recently revealed that this relatively low FAD content is the result of competitive non-covalent binding of ADP into the FAD binding site. The sequence-related human MAO A and B bind one covalently linked FAD molecule per protomer [13]. This suggests that in some proteins covalent flavinylation may have evolved to outcompete ADP binding.

In this study, our goal is to identify the residues that tune the cofactor binding properties of PuO. For this, the crystal structures of wild-type PuO and several mutants have been elucidated. Furthermore, electrospray ionization mass spectroscopy (ESI-MS) is used to determine the FAD and ADP contents and their distribution in the dimeric protein. The structural data, together with enzyme kinetic data, reveal that subtle changes in the FAD binding pocket can significantly (1) increase binding of FAD over ADP, and (2) lead to efficient covalent incorporation of the FAD cofactor.

EXPERIMENTAL PROCEDURES

Chemicals

Oligonucleotides and horse-radish peroxidase were from Sigma-Aldrich. *PfuTurbo* DNA polymerase was from Stratagene. *Escherichia coli* TOP10 from Invitrogen was used as a host for DNA manipulations and protein expression. All other chemicals were of analytical grade. Constructs were sequenced at GATC Biotech (Konstanz, Germany).

Construction of PuO P15I/A394C, PuO A394C/V433M and PuO A394C/A396/ Q431G

The mutants were prepared using the QuikChange site-directed mutagenesis kit from Stratagene. pBAD-PuO_{Rh}A394C [11] was the template. The primers used for this were:

P15I_frw, 5'-GTCGGCGCCGGCATCTCTGGCCTGG-3';

P15I_rv, 5'- CCAGGCCAGAGATGCCGGCGCCGAC-3';

V433M_frw, 5'-GAGGGGTACCAGCACATGGACGGTGCCGTTC-3';

V433M_rv, 5'-GAACGGCACCGTCCATGTGCTGGTACCCCTC-3';

A396T_frw, 5'- CGCGGTTGTTACACGGCGAGCTTCGATC-3';

A396T_rv, 5'- GATCGAAGCTCGCCGTGTAACAACCGCG-3';

Q431G_frw, 5'- GCCGAGGGGTACGGGCACGTCGACGG-3';

Q431G_rv, 5'- CCGTCGACGTGCCCGTACCCCTCGGC-3'.

The sites of mutation are underlined.

Expression and purification

Expression and purification of wild-type PuO and its mutants was performed as described before [8].

FAD covalent incorporation reaction

To monitor FAD-protein covalent bond formation in PuO, enzyme (50 mM Tris-HCl, pH 7.5) was diluted in 50 mM sodium carbonate, pH 10.0 (final pH 8.3) and incubated at 17°C. No exogenous FAD was added. Samples were taken in time and analysed for activity and amount for covalent FAD.

ESI-MS experiments

For nanoflow ESI-MS analysis under native conditions, enzyme samples were prepared in 50 mM ammonium acetate, pH 6.8. For analysis under denaturing conditions, enzyme samples were diluted either in 50% acetonitrile with 0.2% formic acid or in 5% formic acid. Analysis was performed on a LC-T nanoflow ESI orthogonal TOF mass spectrometer (Micro-mass, Manchester, UK). All measurements were performed by operating in the positive ion mode and by using gold-coated needles, made from borosilicate glass capillaries (Kwik-Fil; World Precision Instruments, Sarasota) on a P-97 puller (from Sutter Instruments, Novato). The needles were coated with a gold layer, which was performed by an Edwards Scancoat six Pirani 501 sputter coater (Edwards Laboratories, Milpitas). Mass spectra were calibrated using cesium iodide in water (25 mg/ml).

Analytical methods

Absorbance spectra were recorded in 50 mM Tris-HCl, pH 7.5 or Tris-carbonate, pH 8.3, at 25°C on a PerkinElmer Lambda Bio40 spectrophotometer. Enzyme concentrations were determined by measurement of absorbance at 280 nm using ε_{280}=63370 $M^{-1}cm^{-1}$ (http://www.expasy.ch/sprot/) and recalculated for monomers containing FAD. Oxidase activity was measured by using a coupled horseradish peroxidase assay as described before at pH 8.0 [8]. To measure the non-

covalent FAD concentration (ε_{450}=11.3 $mM^{-1}cm^{-1}$), 5% trichloroacetic acid (TCA) was used to precipitate the protein and the absorbance spectrum of the supernatant was recorded after centrifugation.

Crystallization and structure determination

Crystallization conditions were initially screened using an Oryx8 robot (Douglas Instruments) with 96-well plates. Positive results were obtained in vapor microbatch under a mixture of 70% paraffin – 30% silicon oils with ammonium sulfate and PEG400 precipitants. Conditions were further optimized and crystals of wild-type and mutant enzymes used for structure determination were obtained by sitting-drop vapor diffusion method at 4°C by mixing equal volumes (2-4 µl) of protein and reservoir solutions. Protein solutions consisted of 8-10 mg enzyme/ml in phosphate buffer pH 7.2 whereas precipitant solutions consisted of 2.0 - 2.4 M ammonium sulfate, 0.1 M sodium citrate, and 0.1 M Mes-HCl pH 6.4. Structure of the wild-type protein in complex with the reaction product was obtained by soaking a crystal at 20°C for 1 hour in a solution containing 10 mM 1,4-diaminobutane, 2.5 M ammonium sulfate, 0.1 M sodium citrate, and 0.1 M Mes-HCl pH 6.4.

For data collection, crystals were briefly soaked in a cryoprotectant solution consisting of 20% (v/v) glycerol, 2.5 M ammonium sulfate, 0.1 M sodium citrate, and 0.1 M Mes-HCl pH 6.4. X-ray diffraction data was collected at 100 K at the European Synchrotron Radiation Facility in Grenoble (France). Images were processed using the CCP4 suite programs [14]. The structure of the wild-type enzyme was solved by molecular replacement using the structure of a monomer of MAO B as search model (Protein Data Bank entry 2V5Z). All other PUO structures were solved by molecular replacement employing the wild-type structure as search model. Crystallographic computing and model analysis were performed with COOT [15] and programs of the CCP4 package [14]. Pictures were generated with the program CCP4mg [16]. Cavity calculations were performed with the program VOIDOO [17]. Data collection parameters and final refinement statistics are listed in Table 3.1.

RESULTS

Structural properties of wild-type PuO

PuO is a stable protein which crystallizes quite readily, yielding different crystal forms (Table 3.1). Structure determinations and refinements were done following standard crystallographic protocols, taking advantage of the good quality of the diffraction data which produced excellent electron density maps. All protein residues (except the N-terminal first amino acid and the C-terminal 3-4 residues) have well-defined ordered conformations. Atomic superpositions indicate that the conformations of the crystallographically independent protein subunits are highly conserved across different crystal forms as shown by root-mean-square deviations falling in the range of 0.1-0.3 Å (using Cα atoms). This observation implies that both ligand binding and side-chain mutations cause minimal or no conformational changes with respect to the native wild-type enzyme. On this basis, unless otherwise stated, model analysis was carried out with reference the to A subunit of the ligand-bound structure obtained by soaking the crystals in a putrescine-containing solution (Fig. 3.1A). With the exception of the A394C/A396T/Q431G triple mutant (see below), the flavin ring of FAD exhibits well defined electron densities and was refined with full occupancy (Fig. 3.1B). This indicates that the FAD-bound (rather than ADP-bound) enzyme molecules are preferentially incorporated into the crystals because of more ordered and/or rigid conformations.

The overall structure of PuO is similar to those of human MAO A and MAO B, which represent the closest structural homologues present in the Protein Data Bank [9,18,19] (Fig. 3.1A). Atomic superpositions yield root-mean-square deviations of 1.7 and 1.6 Å for 439 and 445 Cα atoms with 30 and 33% sequence identities, respectively. The comparative analysis indicates that PuO and MAOs share a generally similar backbone conformation with local conformational variations but no large alterations due to insertions/deletions or large shifts of secondary structures. Furthermore, PuO forms the same dimeric structure as found in human MAO B consistent with its elution profile on size-exclusion chromatography and MS data [11].

Despite such a clear relation with human MAOs, analysis of the PuO structure highlights several interesting features, which deserve a closer description. A first intriguing point is the remarkable similarity between the FAD-binding sites

of PuO and human MAO A/B. The conformations of the MAO residues involved in the covalent bond with the flavin are very similar to those of the corresponding region of PuO in which Ala394 replaces the flavinylated Cys of MAOs (Fig. 3.2A). This feature is particularly noticeable because the flavinylated Cys of MAOs is connected to a Tyr residue through a conformationally unfavourable *cis* peptide bond. Such a Cys-Tyr motif of MAOs is replaced by Ala394-Tyr395 in PuO, which, strikingly, exhibits the same *cis* peptide bond. As a result, Ala394 is positioned close to the C8α-methyl group of the flavin, which has profound implications for generating a site for covalent flavinylation in PuO, as described below.

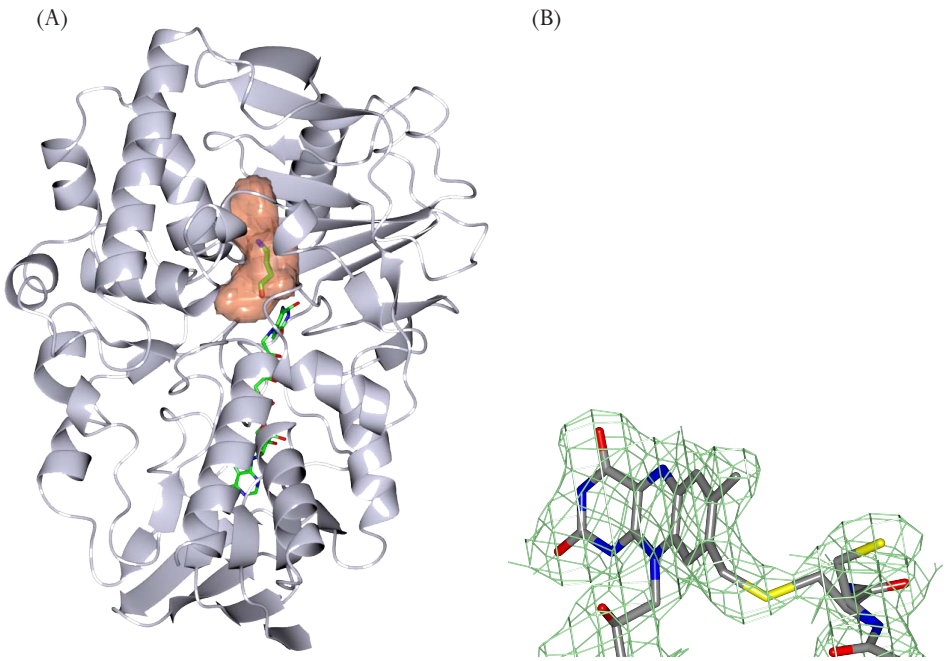

Figure 3.1. Overall structure of PuO and quality of the crystallographic data. **(A)** The protein exhibits the same folding topology as MAOs. FAD and bound 4-aminobutanal are shown as ball-and-sticks with carbons in green, oxygens in red, nitrogens in blue, and phosphorous atoms in magenta. The active site cavity is outlined as a semi-transparent red surface. **(B)** Crystallographic data for the P15I/A394C PuO mutant structure (subunit B). The picture shows the final weighted $2F_o$-F_c electron density map for the flavin ring and the double conformation of Cys394. The contour level is 1σ. Oxygens are in red, nitrogens in blue, phosphorous atoms in magenta, carbons in gray, sulphurs in yellow.

Table 3.1. Crystallographic statistics

	Wild-type	Product-bound	A394C	P15I/A394C	A394C/A396T/Q431G
Space group	$P2_122_1$	$P2_122_1$	$P4_122$	$P2_122_1$	$I2_12_12_1$
Unit cell axes (Å)	197.6, 79.8, 91.7	198.6, 80.6, 92.1	102.0, 102.0, 130.4	198.4, 80.3, 91.4	114.0, 120.4, 171.4
Resolution (Å)	2.0	2.3	1.9	2.5	2.7
R_{merge} (%)¶	9.5 (31.2)	12.8 (39.4)	12.4 (51.2)	9.7 (24.0)	14.6 (59.8)
Completeness (%)¶	99.8 (96.2)	99.8 (99.4)	99.8 (99.4)	99.5 (100.0)	99.8 (99.4)
Unique Reflections	96941	66382	54711	51121	31023
Multiplicity	3.7	3.8	14.5	5.0	4.1
R_{cryst} (%)	17.5	15.7	19.8	18.4	23.0
R_{free} (%)	20.1	19.9	24.2	23.1	27.6
rmsd bond length(Å)	0.014	0.023	0.030	0.022	0.015
rmsd bond angles (°)	1.4	2.0	2.1	1.9	1.6

¶ Data for the highest resolution shell are in parentheses.

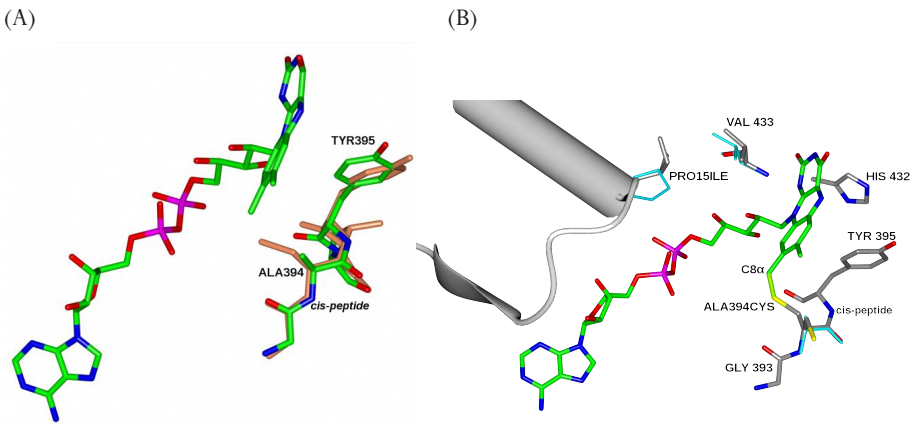

Figure 3.2. Close-up view of the flavin-binding site of PuO in the same orientation as in Fig 3.1. **(A)** Comparison of the structure of wild-type PuO with that of human MAO B. PuO oxygens are in red, nitrogens in blue, phosphorous atoms in magenta, carbons in green, sulphurs in yellow. Gly396, Cys397, Tyr398, and Thr399 of human MAO B are superimposed and show in light brown. **(B)** Structure of the P15I/A394C PuO mutant (subunit B). Oxygens are in red, nitrogens in blue, phosphorous atoms in magenta, protein carbons in grey, and FAD carbons in green. Wild-type Pro15, Ala394, Val433 are superimposed and shown in cyan. Pro15 is located at the N-terminus of the α-helix (shown as cylinder) of the $\beta\alpha\beta$ unit which forms the binding site for the pyrophosphate group of FAD. Ala394 is in contact with the cofactor C8α-methyl group and is engaged in a *cis* peptide link with Tyr395. The Cys in P15I/A394C mutant has a double conformation, one of them (50% occupancy) forming a covalent bond with the flavin.

Another interesting feature concerns the active site. Soaking the crystals in a putrescine-containing solution led to a well-defined electron density peak which can perfectly be fitted by a linear six-atom ligand. We modelled this compound as 4-aminobutanal, assuming that the complex corresponds to the product-bound enzyme (Fig. 3.3). However, we cannot rule out that the crystals contain bound putrescine or a mixture of substrate and product. PuO exhibits a cavity in front of the flavin cofactor with a volume of 300 $Å^3$ (Fig. 3.1A). This cavity has a bipartite shape with approximately equal inner and outer chambers. The inner space is occupied by the 4-aminobutanal, which extends across the cavity along a direction orthogonal to the *re*-face of the flavin ring. As often observed in flavin amine oxidases, the isoalloxazine ring adopts a non-planar distorted conformation [20]. The ligand aldehyde group is in contact with the flavin and is located between the aromatic rings of Tyr395 and His432. These two side chains form an "aromatic sandwich" similar to that of other amine oxidases [20] (Fig. 3.3). The only difference is that PuO has a His/Tyr pair rather than the Phe/Tyr or Tyr/Tyr combination found in polyamine oxidase and in MAOs [9,10,21] although mutagenesis data have shown that human MAO B retains activity even with a His/Tyr pair as found in PuO [22]. The ligand amino group is engaged in H-bonds with the side chain of Glu324 and the carbonyl oxygen of Gly172. These two residues together with Met173 generate the constriction that separates the two chambers of the active site cavity (Fig. 3.1A and 3.3). Of particular relevance is the role of Glu324, which functions as a sort of active site ruler that determines the distance between the flavin and the binding site for the positively-charged amino group of the substrate. This feature is a key element to explain PuO's preference for diamino substrates such as putrescine and cadaverine, in which the two amino groups are separated by four and five carbon atoms, respectively. The shape and size of the outer chamber of the cavity extending beyond Glu324 provides additional space for binding an aliphatic substituent on the distal amino group, which is consistent with the ability of PuO to oxidize spermidine (although less efficiently than putrescine; Fig. 3.1A). In summary, PuO is a typical amine oxidase in which the local conformation and the nature of the residues surrounding the active-site cavity dictate the enzyme preferences for small aliphatic di-amines.

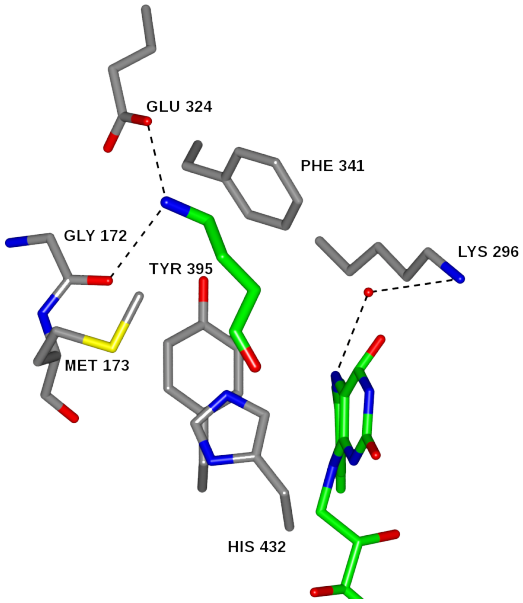

Figure 3.3. Binding of the active-site ligand present in the structure obtained by crystal soaking in a putrescine-containing solution. The ligand has been tentatively modelled as 4-aminobutanal. Oxygens are in red, nitrogens in blue, phosphorous atoms in magenta, protein carbons in grey, FAD carbons in green. H-bonds are shown as dashed lines.

Improving the affinity towards FAD: P15I/A394C and A394C/V433M

In a previous study, the A394C PuO mutant was constructed, purified and characterized [11]. The introduced cysteine is structurally analogous to the Cys that forms a covalent cysteinyl-FAD linkage in MAO (Fig. 3.2A). The mutant was found to retain activity but only a marginal fraction (15%) of the mutant protein contained covalently bound FAD, whereas the rest of FAD was still dissociable (Tables 3.2 and 3.3). In the framework of the present study, we have solved the A394C crystal structure, which turned out to be virtually indistinguishable from that of the wild-type enzyme (Table 1). Cys394 is partly disordered and was modelled in two alternative conformations, with neither of them forming a covalent bond with the flavin. Thus, the crystalline mutant A394C enzyme does not contain covalently bound flavin, at least in a detectable level, which is consistent with the low degree of covalent flavinylation exhibited by the mutant protein (Table 3.2). On the other hand, the A394C structure clearly indicated that a Cys side chain can

be introduced at position 394 without any large structural perturbation, supporting the idea that further amino acid substitutions might render PuO fully capable to efficiently bind FAD in covalent manner.

Table 3.2. Cofactor distribution in PuO variants and amount of FAD covalently bound to the monomers before and after FAD covalent incorporation. The numbers represent an average of at least 3 measurements with standard error less than 5%.

	Cofactor distribution in dimers (%)¶				% PuO monomers with FAD covalently bound‡	
	2ADP	ADP+FAD	2FAD	FAD:ADP ratio	as isolated	48h incubation
wild-type	24	35	41	1.4	0	0
A394C	27	38	35	1.2	22	42
P15I/A394C	0	14	86	13.3	16	35
A394C/V433M	3	26	71	5.2	0	nd
A394C/A396T/Q431G	23	39	39	1.4	40	56

¶The cofactor distribution in dimers was measured with ESI-MS under native conditions.

‡The amount of monomers with FAD covalently bound for freshly isolated enzyme was measured with ESI-MS in denaturing conditions and recalculated for monomers containing FAD and not ADP (based on ESI-MS in native conditions). The fractions of monomers with covalently bound FAD after 48 hours incubation were measured with the TCA method.

nd – not determined

Table 3.3. Steady state kinetic parameters for PuO variants with putrescine as substrate.

	t = 0			t = 48 h		
	k_{cat} [s^{-1}]	K_M [μM]	k_{cat}/K_M [$s^{-1}mM^{-1}$]	k_{cat} [s^{-1}]	K_M [μM]	k_{cat}/K_M [$s^{-1}mM^{-1}$]
wild-type	19.0±0.6	45.4±2.3	419±21	8.8±0.2	17.7±0.4	497±19
A394C	2.4±0.1	19.7±1.5	123±9	2.4±0	9.2±0.6	261±17
P15I/A394C	0.79±0.01	13.4±0	59±1	0.78±0.01	11.8±1.5	67±8
A394C/A396T/Q431G	0.95±0.01	21.0±1.4	45±3	0.83±0.01	13.7±0.3	60±1

Therefore, we generated other mutants to enhance flavin binding and efficiency of covalent linkage formation. To this aim, we compared the PuO structure with those of other dinucleotide-binding domains available through the Protein Data Bank. Generally, these proteins bear low sequence homologies but all share a

GXGXXG phosphate-binding loop which connects the β-strand to the α-helix of the characteristic βαβ unit [23] (Fig. 3.2B). This dinucleotide-binding motif is not only found in FAD-binding proteins but is also present in e.g. ADP-, ATP-, and NAD(P)-binding proteins [24]. Inspection of the residues encompassing the dinucleotide-binding motif in PuO reveals an atypical residue, Pro15, next to the second conserved Gly. Sequence comparisons with structurally-related FAD-containing amine oxidases reveal that, in most cases, a bulky hydrophobic residue (Phe, Leu or Ile) is found at this position [25]. These observations prompted us to determine whether this residue may play a role in the unique cofactor binding behaviour of PuO, which binds FAD and ADP in a 1:1 ratio (Table 3.2). Thus, P15I/A394C PuO double mutant was produced and its cofactor binding characteristics were analysed by native ESI-MS [26]. Interestingly, the resulting spectrum reveals only two major dimeric species of 99,801 Da and 100,158 Da (Fig. 3.4). Minor amounts of some other PuO species (3 kDa larger) were observed which are likely to be a result of translation by *E. coli* using an alternative stop codon as observed for wild-type PuO [8,11]. Analysis of the data showed that the majority of the protein (86%, theoretical mass of 100,152 Da) is present as a dimer containing two FAD molecules (Table 3.2). The other dominant species (14%, 99,801 Da) corresponds to dimeric PuO containing one FAD and one ADP (theoretical mass of 99,794 Da). No peaks corresponding to apo dimers or dimers with two ADP molecules bound were found. The calculated FAD to ADP ratio is 9-10 times higher than the one for wild-type PuO confirming that the N-terminal Rossmann-fold domain plays a crucial role in facilitating effective FAD binding (Table 3.2). On the other hand, the P15I/A394C protein did not exhibit any improvement in covalent incorporation of the cofactor since only 16% of the incorporated FAD molecules are covalently bound to the protein (although this values increases to 36% upon prolonged incubation of the protein for two days; see below and Table 3.2 for details).

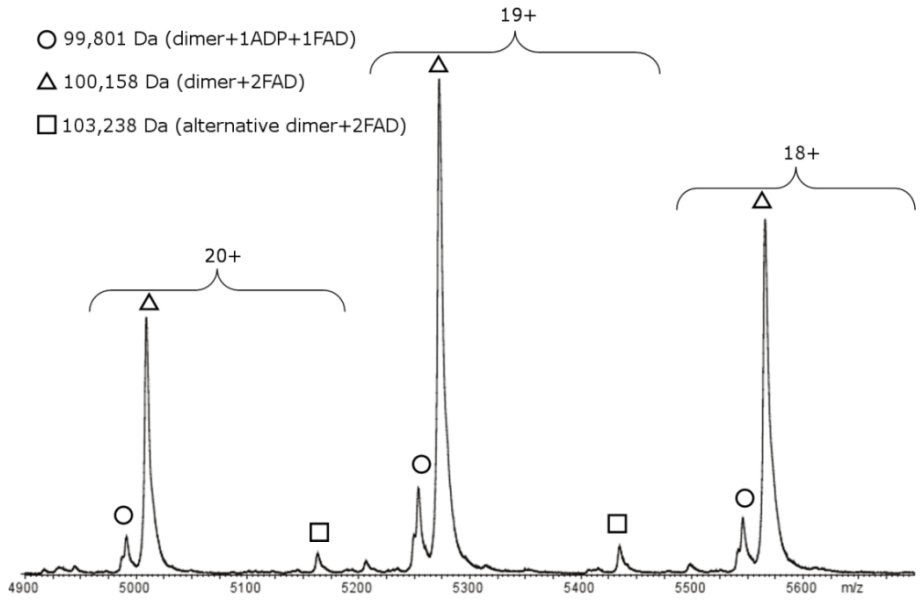

Figure 3.4. Mass spectrum of P15I/A394C PuO in its native state. Observed enzyme species are indicated with symbols and corresponding masses. Alternative dimer is a dimer with alternative stop codon, as discussed in results.

To evaluate the conformational effects of the P15I mutation, the crystal structure of P15I/A394C PuO was determined (Fig. 3.2B). The mutant is virtually identical to the wild-type enzyme with Ile15 overlapping the position of Pro15. The electron density of subunit B (the crystals contain two subunits in the asymmetric unit) indicates that a significant fraction of the protein molecules contain Cys394 covalently bound to the flavin C8α atom (about 50% as judged from the refined atomic occupancy values; Fig. 3.1B). Crystals growth requires 2-3 days, implying the formation of the covalent bond can take place during crystallization. This finding allowed us to visualize the actual presence of a covalent bond between the protein and the cofactor, clearly supporting the idea that covalent bond formation with the flavin is not associated to any significant conformational alterations.

A minor but striking structural change that could be observed in the P15I/A394C structure involves Val433, which is pushed by 0.3 Å towards the N1-C2=O2 locus of the flavin ring, to establish a closer interaction which may be the cause for the improved FAD binding (Fig. 3.2B). Given that in MAO B, a Met is present at the position homologous to Val433 of PuO, we decided to generate A394C/V433M.

We found that this double PuO mutant has indeed a significantly increased FAD:ADP ratio when compared to wild-type and A394C proteins (Table 3.2). This observation further supports the idea that minor alterations in the flavin environment can significantly alter its affinity to the protein cofactor site and change the levels of FAD *versus* ADP incorporation.

Improved covalent FAD incorporation: A394C/A396T/Q431G

To identify residues that hamper effective covalent flavinylation in PuO, we carefully analysed the differences in active sites of PuO and MAO B. According to the proposed self-catalytic mechanism of covalent flavin attachment, the covalent coupling is initiated by proton abstraction from the C8α-methyl group of FAD (Fig. 3.2) and/or from the residue forming the covalent bond with the flavin, suggesting that these deprotonations might limit covalent flavinylation in PuO [3,7]. Structural comparisons indicated that a Thr (Thr398) at a distance of 5.0 Å from the C8α-methyl group of flavin in MAO B is replaced by an Ala (Ala396) in PuO (Fig. 3.5). A Thr at this position may facilitate covalent flavinylation by creating a more polar environment and/or a H-bonding network that favours deprotonation of Cys394 or flavin C8α [9]. However, further inspection of the PuO crystal structure reveals that a Thr side chain in position 396 would probably cause an unfavourable contact with the neighbouring polar Gln431, which is a Gly in MAO-B. Therefore, we generated a triple mutant (A394C/A396T/Q431G) which was comparatively analysed by ESI-MS under denaturing conditions to quantify covalent flavinylation. Figure 3.6 shows the ESI-MS spectra of freshly purified wild-type, A394C, and A394C/A396T/Q431G proteins. Wild-type PuO shows only one species corresponding to the mass of PuO minus the N-terminal methionine (49,244 Da, the theoretical value). The mass spectrum of A394C reveals instead the presence of two species: 49,275 Da and 50,058 Da, corresponding to the calculated values of 49,275 Da and 50,060 Da for A394C without and with covalently bound FAD, respectively. Likewise, the mass spectrum of the denatured A394C/A396T/Q431G PuO also shows two major species: 49,239 Da and 50,024 Da, corresponding to the theoretical 49,234 Da and 50,019 Da, expected for the non-covalent and covalent forms of the mutant protein, respectively. However, the distribution of the two species differs between A394C and A394C/A396T/Q431G (Table 3.2): the triple mutant has a significantly higher degree of covalent FAD

binding (40% covalent FAD) when compared to A394C PuO (22% covalent FAD). Clearly, the triple mutation favours formation of the covalent bond between Cys394 and FAD. However, the protein exhibits an overall (covalent and non-covalent) level of FAD incorporation similar to that of the wild-type protein as gathered from the similar values of FAD:ADP ratio measured by native ESI-MS (Table 3.2). This implies that the increased efficiency of flavin-protein covalent bond formation is not accompanied by an enhancement in affinity towards FAD compared to ADP.

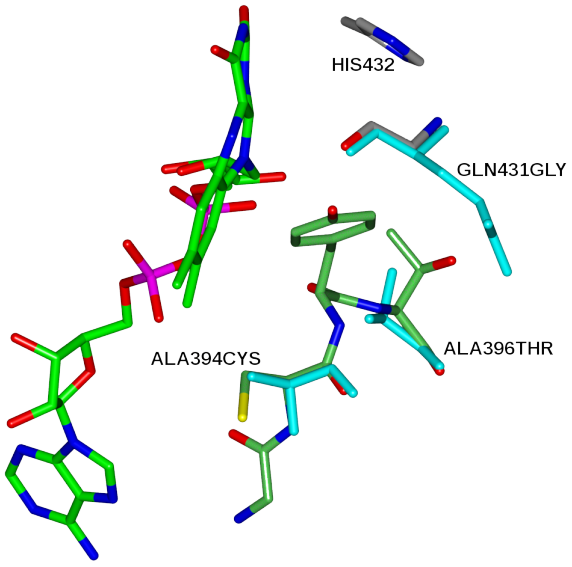

Figure 3.5. Comparison of the A394C/A396T/Q431G structure with that of the wild-type PuO (subunit B). Oxygens are in red, nitrogens in blue, and phosphorous atoms in magenta. The mutant carbon atoms are in grey whereas FAD carbons are in green. Wild-type Ala394, Ala396, and Gln431 are shown in cyan.

As for the other enzyme variants, the triple mutant crystal structure exhibits minor conformational changes, with the newly introduced side chains apparently causing little perturbations (Fig. 3.5). A394C/A396T/Q431G was the only mutant protein to show somewhat less well-defined electron density for the flavin ring. This implies that the flavin site is not fully occupied (i.e. the crystalline proteins are partly in the ADP-bound form) or that there might be some disorder in the flavin conformation. Nevertheless, the electron density for subunit B shows that there is a partial (approximately 50% occupancy) covalent attachment of the C8α-methyl

group of the flavin to Cys394, in keeping with the enhanced efficiency of covalent flavinylation exhibited by the mutant, as indicated by ESI-MS spectra (Fig. 3.6).

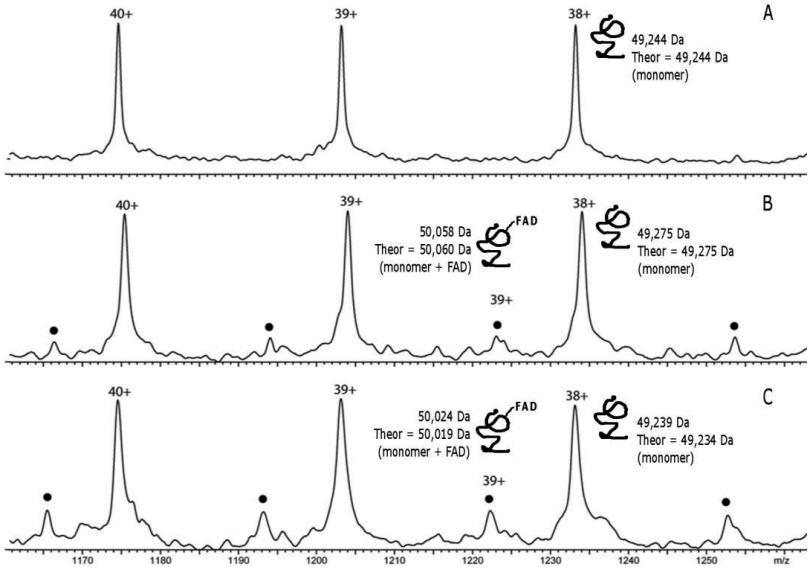

Figure 3.6. Mass spectra of denatured wild-type (A), A493C (B), and A394C/A396T/Q431G (C) PuO. Observed enzyme species with covalently bound FAD marked with a dot. Theoretical masses correspond to a monomer mass of the PuO variant minus N-terminal methionine.

Kinetics of FAD incorporation and enzymatic activities

In order to assess the time-dependence of C8α-*S*-cysteinyl bond formation in PuO mutants, the covalent incorporation of FAD was monitored over time. To facilitate deprotonation and hence flavinylation the experiments were performed at relatively high pH values. Enzyme stocks (210-460 µM in 50 mM Tris-HCl, pH 7.5) were diluted in a 1:1 ratio with 50 mM sodium carbonate buffer, pH 10.0, containing 0.04% sodium azide. The enzyme solutions were incubated at 17°C for two days and the covalent FAD content and steady-state kinetics were measured at the beginning and at the end of the incubation (Table 3.3). In order to determine the amount of FAD covalently bound to the enzymes after 48 hours, the TCA precipitation method was used. Flavin covalently bound to the protein will co-precipitate whereas holo-proteins with non-covalently bound FAD will

release the cofactor in solution upon denaturation. In this way, it is possible to estimate the ratio between covalent and non-covalent holoenzyme concentrations. As can be seen from Table 3.3, PuO mutants generally increase covalent incorporation of FAD during the incubation time, which is not observed for wild-type PuO. However, the process is very slow and still not all FAD is covalently attached. The A394C/A396T/Q431G PuO is the most effective in flavin-protein covalent bond formation. After two days it covalently incorporates more than half of the FAD. To further confirm this result, the mutant incubated for three days was analysed by ESI-MS under denaturing conditions. The spectrum showed again two major peaks with the masses of 49,239 Da and 50,022 Da, corresponding to the theoretical (see above) masses of the monomer and the monomer with covalently bound FAD. The corrected value (the ratio between FAD and ADP did not change) for monomers with covalently bound FAD was 66%, which is in line with the value obtained with the TCA precipitation method (56% after two days). Steady-state kinetic parameters for putrescine were also determined before and after the incubation of the PuO variants (Table 3.3). It is apparent that wild-type PuO is more active than the mutants. Its catalytic efficiency (k_{cat}/K_M) is almost four times higher than that of A394C mutant and eight times higher than those of the two other mutants. Interestingly, after a two-day incubation, the k_{cat} of wild-type PuO is half the original value, whereas it stays at the same level in the mutants. This finding suggests that covalent binding of FAD increases protein stability and maintenance of enzyme activity.

DISCUSSION

The crystal structures of PuO reveal that the enzyme possesses all the characteristic features of flavin-amine oxidases; an aromatic sandwich, a conserved pattern of interactions with the cofactor, a distorted non-planar flavin, and a closed cavity for substrate binding, whose shape and H-bonding properties determine the substrate specificity [9,10,20] (Fig. 3.1-3.3). Compared to human MAOs, the most extensively studied members of the amine oxidase family, an interesting feature of PuO is that it lacks a covalent linkage between protein and the flavin ring. However, this property is not associated to any structural alterations within the cofactor site which is very similar to that of human MAOs. This includes

the conservation of the *cis* peptide bond of the Tyr residue that is part of the aromatic cage forming the binding site for the substrate amino group – i.e. the site oxidation [20]. This finding suggests that this *cis* peptide is mainly necessary to attain the proper geometry in the active-site cavity and flavin binding region rather than for covalent flavinylation (Fig. 3.2). On the other hand, such geometry is perfectly tailored to engage the flavin C8α -methyl group in a covalent bond with the protein. This is a "natural" event in MAOs whereas it can be "forced" to occur in PuO by site-directed mutagenesis, taking advantage of the apparent rigidity of the PuO's cofactor site, which is seemingly pre-organized for the effective introduction of the covalent protein-flavin bond. Indeed, the engineered PuO mutants, despite the introduced mutations, remain active, which shows that insertion/deletion of the covalent linkage is not essential for activity as found in other covalent flavoenzymes including MAO A [2,27].

A hypothesis underlying our studies was that by covalent tethering of FAD, the enzyme could fully incorporate the flavin cofactor, outcompeting the binding of ADP. However, the partially introduced covalent FAD did not result in any significant increase in the FAD incorporation into the enzyme (Table 3.2). This finding fully supports the proposal by Zhou et al. that the dinucleotide-binding motif provides a topological dock for the initial binding of FAD. Only after such a binding event, covalent bond formation can occur [28]. Therefore, the role of covalent flavinylation does not seem to be that of favouring incorporation of FAD compared to other mono- or di-nucleotides that are potentially able to bind to the cofactor site on the protein.

Our studies on PuO also support the idea that covalent flavinylation is a self-catalytic process that critically depends on a proper FAD microenvironment [7,29-34]. Indeed, the double P15I/A394C mutant, which exhibits the highest FAD:ADP ratio, shows instead a poor level of covalent FAD binding, suggesting a sub-optimal environment for covalent bond formation. Conversely, by introducing mutations predicted to alter the flavin C8α environment (A394C/A396T/Q431G) the degree of covalent flavin attachment was increased (Table 3.2). The *in vitro* process of covalent flavinylation has been studied for several flavoenzymes by expressing the respective apo proteins in riboflavin auxotrophic expression strains. This has enabled the monitoring of the time-dependent covalent incorporation of flavin cofactors. Apo forms of these natural covalent flavoenzymes covalently anchor their FAD within several minutes (vanillyl-alcohol oxidase [35] and

monomeric sarcosine oxidase [36]) or even hours (dimethylglycine dehydrogenase [37]). Therefore, the slow formation of the covalent linkage observed in the PuO triple mutant is not exceedingly far from the slow flavinylation rates observed in certain natural covalent flavoproteins (Table 3.2). Altogether, these findings demonstrate that structure-based design of covalent flavinylation is feasible although it may require several amino acid replacements, not necessarily confined to region in direct contact with the flavin C8α. Employment of flavoproteins for biocatalytic processes is often difficult because of their tendency to release FAD/FMN in the conditions of relevance for biocatalytic applications. The possibility of engineering flavoproteins to incorporate the cofactor through a covalent linkage will be critical to make progress in this area.

REFERENCES

[1] O. Dym, D. Eisenberg, Sequence-structure analysis of FAD-containing proteins, Protein Sci. 10 (2001) 1712-1728.

[2] D.P. Heuts, N.S. Scrutton, W.S. McIntire, M.W. Fraaije, What's in a covalent bond? On the role and formation of covalently bound flavin cofactors, FEBS J. 276 (2009) 3405-3427.

[3] M. Mewies, W.S. McIntire, N.S. Scrutton, Covalent attachment of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to enzymes: the current state of affairs, Protein Sci. 7 (1998) 7-20.

[4] J. Kim, J.H. Fuller, V. Kuusk, et al., The cytochrome subunit is necessary for covalent FAD attachment to the flavoprotein subunit of p-cresol methylhydroxylase, J. Biol. Chem. 270 (1995) 31202-31209.

[5] R. Brandsch, V. Bichler, Autoflavinylation of apo6-hydroxy-D-nicotine oxidase, J. Biol. Chem. 266 (1991) 19056-19062.

[6] K.M. Robinson, B.D. Lemire, Covalent attachment of FAD to the yeast succinate dehydrogenase flavoprotein requires import into mitochondria, presequence removal, and folding, J. Biol. Chem. 271 (1996) 4055-4060.

[7] P. Trickey, M.A. Wagner, M.S. Jorns, F.S. Mathews, Monomeric sarcosine oxidase: structure of a covalently flavinylated amine oxidizing enzyme, Structure. 7 (1999) 331-345.

[8] E.W. van Hellemond, M. van Dijk, D.P. Heuts, D.B. Janssen, M.W. Fraaije, Discovery and characterization of a putrescine oxidase from *Rhodococcus erythropolis* NCIMB 11540, Appl. Microbiol. Biotechnol. 78 (2008) 455-463.

[9] D.E. Edmondson, C. Binda, J. Wang, A.K. Upadhyay, A. Mattevi, Molecular and mechanistic properties of the membrane-bound mitochondrial monoamine oxidases, Biochemistry. 48 (2009) 4220-4230.

[10] K.E. Atkin, R. Reiss, V. Koehler, et al., The structure of monoamine oxidase from *Aspergillus niger* provides a molecular context for improvements in activity obtained by directed evolution, J. Mol. Biol. 384 (2008) 1218-1231.

[11] E.W. van Hellemond, H. Mazon, A.J. Heck, et al., ADP competes with FAD binding in putrescine oxidase, J. Biol. Chem. 283 (2008) 28259-28264.

[12] R.J. DeSa, Putrescine oxidase from *Micrococcus rubens*. Purification and properties of the enzyme, J. Biol. Chem. 247 (1972) 5527-5534.

[13] E.B. Kearney, J.I. Salach, W.H. Walker, et al., The covalently-bound flavin of hepatic monoamine oxidase. 1. Isolation and sequence of a flavin peptide and evidence for binding at the 8alpha position, Eur. J. Biochem. 24 (1971) 321-327.

[14] Collaborative Computational Project, Number 4, The CCP4 suite: programs for protein crystallography, Acta Crystallogr. D Biol. Crystallogr. 50 (1994) 760-763.

[15] P. Emsley, B. Lohkamp, W.G. Scott, K. Cowtan, Features and development of Coot, Acta Crystallogr. D Biol. Crystallogr. 66 (2010) 486-501.

[16] L. Potterton, S. McNicholas, E. Krissinel, et al., Developments in the CCP4 molecular-graphics project, Acta Crystallogr. D Biol. Crystallogr. 60 (2004) 2288-2294.

[17] G.J. Kleywegt, T.A. Jones, Detection, delineation, measurement and display of cavities in macromolecular structures, Acta Crystallogr. D Biol. Crystallogr. 50 (1994) 178-185.

[18] S.Y. Son, J. Ma, Y. Kondou, M. Yoshimura, E. Yamashita, T. Tsukihara, Structure of human monoamine oxidase A at 2.2-A resolution: the control of opening the entry for substrates/inhibitors, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 5739-5744.

[19] H.M. Berman, J. Westbrook, Z. Feng, et al., The Protein Data Bank, Nucleic Acids Res. 28 (2000) 235-242.

[20] C. Binda, A. Mattevi, D.E. Edmondson, Structure-function relationships in flavoenzyme-dependent amine oxidations: a comparison of polyamine oxidase and monoamine oxidase, J. Biol. Chem. 277 (2002) 23973-23976.

[21] C. Binda, R. Angelini, R. Federico, P. Ascenzi, A. Mattevi, Structural bases for inhibitor binding and catalysis in polyamine oxidase, Biochemistry. 40 (2001) 2766-2776.

[22] M. Li, C. Binda, A. Mattevi, D.E. Edmondson, Functional role of the "aromatic cage" in human monoamine oxidase B: structures and catalytic properties of Tyr435 mutant proteins, Biochemistry. 45 (2006) 4775-4784.

[23] R.K. Wierenga, De Maeyer, Marc C. H., W.G.J. Hol, Interaction of pyrophosphate moieties with alpha-helixes in dinucleotide-binding proteins, Biochemistry (N.Y.). 24 (1985) 1346-1357.

[24] O. Vallon, New sequence motifs in flavoproteins: evidence for common ancestry and tools to predict structure, Proteins. 38 (2000) 95-114.

[25] P. Trickey, J. Basran, L.Y. Lian, et al., Structural and biochemical characterization of recombinant wild type and a C30A mutant of trimethylamine dehydrogenase from *Methylophilus methylotrophus* (sp. W(3)A(1)), Biochemistry. 39 (2000) 7678-7688.

[26] R.H. van den Heuvel, A.J. Heck, Native protein mass spectrometry: from intact oligomers to functional machineries, Curr. Opin. Chem. Biol. 8 (2004) 519-526.

[27] J.R. Miller, D.E. Edmondson, Influence of flavin analogue structure on the catalytic activities and flavinylation reactions of recombinant human liver monoamine oxidases A and B, J. Biol. Chem. 274 (1999) 23515-23525.

[28] B.P. Zhou, D.A. Lewis, S.W. Kwan, C.W. Abell, Flavinylation of monoamine oxidase B, J. Biol. Chem. 270 (1995) 23653-23660.

[29] C. Walsh, Flavin Coenzymes: At the Crossroads of Biological Redox Chemistry, Acc. Chem. Res. 13 (1980) 148-155.

[30] I. Efimov, W.S. McIntire, A study of the spectral and redox properties and covalent flavinylation of the flavoprotein component of *p*-cresol methylhydroxylase reconstituted with FAD analogues, Biochemistry. 43 (2004) 10532-10546.

[31] J.W. Koetter, G.E. Schulz, Crystal structure of 6-hydroxy-D-nicotine oxidase from *Arthrobacter nicotinovorans*, J. Mol. Biol. 352 (2005) 418-428.

[32] W. Weyler, Y.P. Hsu, X.O. Breakefield, Biochemistry and genetics of monoamine oxidase, Pharmacol. Ther. 47 (1990) 391-417.

[33] I. Hirashiki, F. Ogata, A. Ito, Rat monoamine oxidase B expressed in *Escherichia coli* has a covalently-bound FAD, Biochem. Mol. Biol. Int. 37 (1995) 39-44.

[34] G. Lu, T. Unge, J.B. Owera-Atepo, J.C. Shih, J. Ekblom, L. Oreland, Characterization and partial purification of human monoamine oxidase-B expressed in *Escherichia coli*, Protein Expr. Purif. 7 (1996) 315-322.

[35] J. Jin, H. Mazon, R.H. van den Heuvel, A.J. Heck, D.B. Janssen, M.W. Fraaije, Covalent flavinylation of vanillyl-alcohol oxidase is an autocatalytic process, FEBS J. 275 (2008) 5191-5200.

[36] A. Hassan-Abdallah, R.C. Bruckner, G. Zhao, M.S. Jorns, Biosynthesis of covalently bound flavin: isolation and *in vitro* flavinylation of the monomeric sarcosine oxidase apoprotein, Biochemistry. 44 (2005) 6452-6462.

[37] C. Brizio, R. Brandsch, M. Douka, R. Wait, M. Barile, The purified recombinant precursor of rat mitochondrial dimethylglycine dehydrogenase binds FAD *via* an autocatalytic reaction, Int. J. Biol. Macromol. 42 (2008) 455-462.

CHAPTER 4

TURNING A MONOCOVALENT FLAVOPROTEIN INTO A BICOVALENT FLAVOPROTEIN BY STRUCTURE-INSPIRED MUTAGENESIS

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ABSTRACT

A recently discovered class of bicovalent flavoproteins is an interesting group of enzymes because of their unusual cofactor binding mode, their open active sites and bulky substrates they can accept. By a sequence comparison study we have identified a conserved sequence region in bicovalent flavoproteins that is different from monocovalent flavoproteins. Based on this and the available structural information we have designed mutants of the prototype monocovalent flavoprotein, 6-hydroxy-D-nicotine oxidase (6HDNO), in order to introduce a second cofactor-protein linkage. Two amino acid replacements, namely histidine 130 to a cysteine and leucine 138 to a histidine, were sufficient to create a bicovalent 6HDNO. The introduced cysteine forms a covalent bond with FAD as found in natural bicovalent flavoproteins, while the second mutation was found to be essential to facilitate formation of the cysteinyl linkage. This points to an important role of the introduced histidine in stabilizing a negative charge of the isoalloxazine ring during covalent flavinylation. The His130Cys/Leu138His 6HDNO is still active and shows a higher midpoint redox potential when compared to wild-type 6HDNO. This is in agreement with the previous studies that have shown that bicovalent flavoenzymes have extremely high redox potentials.

INTRODUCTION

Since the discovery of the first "yellow" enzyme in the 1930's [1], the number of known flavoproteins is constantly growing. The yellow colour in flavoproteins originates from the flavin cofactor, namely flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), with FAD being the most common. Enzymes utilize cofactors for redox reactions, because the chemical functionality of amino acids is limited. Flavin-dependent enzymes are very diverse, because they catalyse a great number of redox and non-redox reactions displaying various mechanistic pathways [2]. Moreover, they do not have one common mode of cofactor binding. In most cases the flavin cofactor is tightly, but non-covalently associated with the protein, and can be regarded as a prosthetic group. Yet, approximately 10% of all flavoproteins have been confirmed to form a covalent bond with the flavin [3]. This variable mode of flavin binding is quite unique because most cofactors bind either in a noncovalent or covalent manner. The covalent attachment of FMN is rather rare compared to FAD and occurs *via* the 8α-position (to a histidine) or *via* the 6-position (to a cysteine) of the isoalloxazine ring [4] or *via* the phosphate group (to a threonine) [5,6]. Covalent binding of FAD typically involves the 8α-position that binds to a histidine, a cysteine, a tyrosine or an aspartate [4,7]. A more complex type of covalent FAD tethering was discovered in 2005 where FAD is bicovalently bound *via* 6-*S*-cysteinyl and 8α-*N*1-histidyl linkages [8]. Since then, several more bicovalent flavoproteins have been identified [8-16]. Detailed biochemical studies have provided information about the role of (bi)covalent flavin binding [10,17-19]. It has been shown that a covalent FAD-protein linkage significantly influences the redox potential of the enzyme, thereby boosting the oxidative power of the respective flavoenzyme. This is clearly illustrated by the observation that bicovalent flavoenzymes show the highest redox potentials among all known flavoproteins [20]. Other effects of covalent flavin attachment include enhancement of protein stability, prevention from cofactor release and protection from flavin deactivation [20].

The vanillyl-alcohol oxidase (VAO) family of flavoproteins consists of enzymes with a conserved FAD-binding domain and a covalent flavin binding mode is relatively common in this group [21,22]. It is worth noting that all bicovalent flavoproteins belong to the VAO family. One of the first covalent VAO flavoenzymes identified was 6-hydroxy-D-nicotine oxidase (6HDNO) from *Arthrobacter nicotinovorans* (Fig. 4.1A) [23-25]. This bacterial enzyme takes part in a nicotine degradation

pathway (Fig. 4.1B) and is a monomeric enzyme containing a histidyl-bound FAD. The last feature distinguishes 6HDNO from its enantioenzyme, 6-hydroxy-L-nicotine oxidase (6HLNO) from the same organism. 6HLNO binds FAD in a non-covalent manner, indicating that the two enzymes are not related at the protein level. 6HDNO was the first flavoenzyme for which covalent FAD incorporation to apo protein was demonstrated *in vitro* [26]. As a result, it has been extensively studied as a prototype for a covalent flavoprotein. From these and other studies on covalent flavoproteins, it can be concluded that the process of covalent attachment of FAD is generally self-catalytic [4,27-31], with the exception of only a few flavin-dependent enzymes where the presence of a helper protein has been shown to be necessary [6,32-34]. With regard to bicovalent flavoproteins, relatively little is known about the mechanism of bicovalent flavinylation, though it has been demonstrated that the formation of the two covalent attachments is self-catalytic and independent from each other [10,19,35].

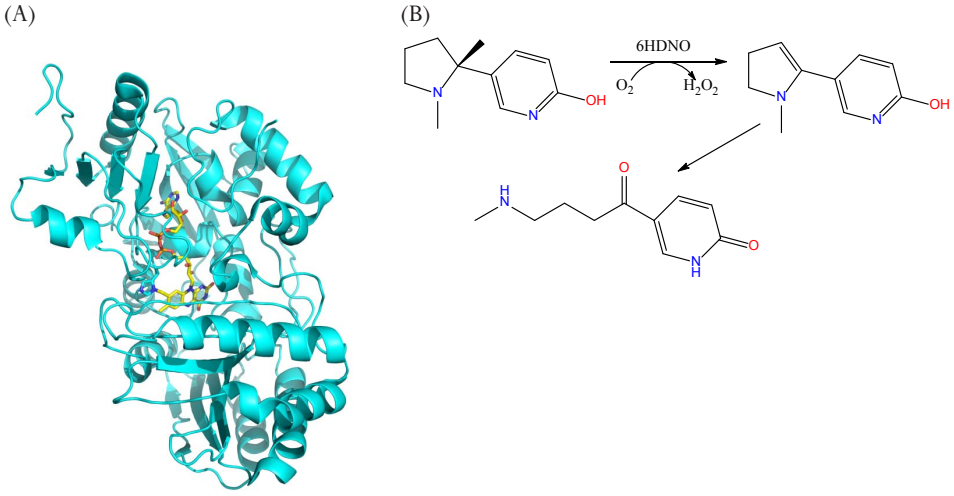

Figure 4.1. (A) Structure of 6HDNO in which the covalently bound FAD cofactor is shown in yellow (PDB:2BVF). **(B)** 6HDNO catalyses the oxidation of the pyrrolidine ring of 6-hydroxy-D-nicotine (top left) to 6-hydroxy-*N*-methylmyosmine (top right), which in turn decays spontaneously by hydration, ring opening and tautomerization to a ketone.

Most of the research on the mechanism and effects of covalent flavinylation has focused on creating protein mutants unable to form a covalent bond with FAD, either by using flavin analogues or by engineering mutants. In a recent study, we

have shown that an opposite approach is also viable. By structure-inspired site-directed mutagenesis we succeeded in introducing a covalent cysteinyl-FAD bond in a non-covalent flavoprotein, putrescine oxidase [36,37]. Here we present an engineered 6HDNO mutant, which is able to form a 6-*S*-cysteinyl bond with FAD, in addition to the 8α-*N*1-histydyl bond already present in the wild-type 6HDNO. We also show how the additional cofactor attachment, rendering an engineered bicovalent flavoprotein, changes the properties of the enzyme. This work contributes to existing knowledge on bicovalent flavinylation. The obtained insights will also help to engineer noncovalent flavoproteins into flavoproteins with a covalently anchored flavin cofactor. The ability to design such artificial covalent flavoproteins will support engineering of robust biocatalysts.

EXPERIMENTAL PROCEDURES

Chemicals

Oligonucleotides were form Sigma and EuroFins. *PfuTurbo* DNA polymerase was from Stratagene. *Escherichia coli* TOP10 from Invitrogen was used as a host for DNA manipulations and protein expression. L-arabinose was from Biosynth. 6-hydroxy-D-nicotine was a kind gift from prof. Roderich Brandsch. Methylene blue, pyocyanin, xanthine, xanthine oxidase, 5,5'dithiobis (2-nitrobenzoic acid) and DL-dithiothreitol were from Sigma-Aldrich. Resorufin was from TCI. All other chemicals were of analytical grade. Constructs were sequenced at GATC Biotech (Konstanz, Germany).

Construction of mutants

pBAD-6HDNO His130Cys and His130Cys/Leu138His were prepared using the QuikChange Site-Directed Mutagenesis kit from Stratagene and pBAD-6HDNO as the template. The primers used for that were the following:
His130Cys_frw, 5'- GTCACCGGCATGTGTCCTAAGGTCGG-3';
His130Cys_rev, 5'- CCGACCTTAGGACACATGCCGGTGAC-3';
Leu138His_frw, 5'- GGTTCTGTGGACACGCGCTCAATGGG-3';
Leu138His_rev, 5'- CCCATTGAGCGCGTGTCCACAGAACC-3'.
The sites of mutation are underlined.

Expression and purification

6HDNO wild-type, His130Cys and His130Cys/Leu138His were expressed in *E. coli* TOP10 cells containing the corresponding pBAD-6HDNO vector. The cells were cultivated for 24 hours at 30°C in terrific broth medium containing 50 µg/ml ampicillin and 0.2% (w/v) L-arabinose. Cells were harvested by centrifugation for 15 min at 6000 rpm at 4°C. The supernatant was discarded and the pellet was resuspended in 30 ml of 20 mM KH_2PO_4 pH 7.5 and sonicated for 10 min in order to break the cells. The cell debris was discarded by centrifugation for 40 min at 15000 rpm at 4°C and the resulting extract was subsequently applied on a Q-Sepharose anion exchange column. Unbound proteins were washed from the column with 20 mM KH_2PO_4 pH 7.5 and 6HDNO was eluted from the column by increasing the KCl concentration. The buffer of eluted 6HDNO fraction was changed to 20 mM KH_2PO_4 pH 7.5, 500 mM NaCl, 15 mM imidazole using an Amicon Ultra-15 Centrifugal Filter Unit with an Ultracel-30 membrane from Merck Millipore. The enzyme fraction was applied on a Ni-Sepharose High Performance column from GE Healthcare and incubated at 4°C for 1.5 h. The unbound fractions were released and the column was washed with 20 mM KH_2PO_4 pH 7.5, 500 mM NaCl, 30 mM imidazole. 6HDNO was eluted with 20 mM KH_2PO_4 pH 7.5, 500 mM NaCl, 300 mM imidazole and desalted on a PD-10 column (GE Healthcare Life Sciences) using 20 mM KH_2PO_4 pH 7.5, 150 mM NaCl.

Cysteine determination

The number of sulfhydryl groups of unfolded 6HDNO variants was determined according to the method of Ellman [38] with the modifications of Habeeb [39]. The assay was performed on 1-5 µM 6HDNO variants in 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% SDS with a 25-times molar excess of 5,5'dithio-bis (2-nitrobenzoic acid) (DTNB). The absorbance of released 5-thio-2-nitrobenzoate anion (TNB) was measured at 412 nm, using $\varepsilon_{412} = 8.4\ mM^{-1}\ cm^{-1}$ determined with DL-dithiothreitol (DTT) in the same buffer, on a PerkinElmer Lambda Bio40 spectrophotometer.

Analytical methods

Sequence alignment was performed with ClustalW at EBI [40,41]. Solvent accessible surface calculations were performed by using the GETAREA server at http://curie.utmb.edu/area_man.html [42]. For structural visualization and inspection, PyMol was used (Schrödinger, LLC). Flavin absorbance spectra of purified 6HDNO variants were recorded in 50 mM Tris-HCl pH 7.5 at 25 °C, before and after addition of 0.1 % (w/v) SDS, on a PerkinElmer Lambda Bio40 spectrophotometer. Enzyme concentrations were determined by measuring absorbance at 280 nm using ε_{280} = 47245 $M^{-1}cm^{-1}$ [43]. Oxidase activity and steady-state kinetic parameters were determined at 30 °C by spectrophotometric measurement of product formation, as described previously [44], using different concentrations of D-6-hydroxynicotine as a substrate. The data were fitted with SigmaPlot12.0 (Systat Software, Inc.), using the Michaelis-Menten equation or the Michaelis-Menten equation with inhibition by substrate (Eq. 4.1).

$$V = \frac{k_{cat}*[S]}{K_M+[S](1+\frac{[S]}{K_i})} \quad \text{(Eq. 4.1)}$$

To confirm the presence of covalently bound flavin, 5% trichloroacetic acid (TCA) was used to precipitate the protein and the pellet and supernatant were assessed visually and/or spectrophotometrically. The in-gel flavin fluorescence was also confirmed in SDS-PAGE gel after purification of the protein. Performic acid was prepared according to [45]. The stability of 6HDNO variants was measured with the ThermoFAD [46] and ThermoFluor [47] methods using 10 µM enzyme. Fluorescence measurements of native and unfolded 6HDNO variants were performed on 10 µM protein in 50 mM Tris-HCl pH 7.6, 150 mM NaCl, without or with 1% SDS, respectively, on a Synergy Mx Multi-Mode Reader (BioTek). The flavoprotein samples were excited at 450 ±13.5 nm and the 800-480 nm emission spectra were collected. The intensity of fluorescence was compared at the emission maximum, 522 nm.

Redox potentials were measured by using the method described by Massey [48] in 20 mM KH_2PO_4 pH 7.5, 150 mM NaCl at 25 °C, with one of the following dyes: methylene blue (+11 mV), pyocyanine (-38 mV) or resorufin (-51 mV). A septum tight cuvette containing 6HDNO variant (10 µM), xanthine (400 µM), benzyl viologen (10 µM) and redox dye (10 µM) was made anaerobic by flushing

with argon. The reaction was started by adding a catalytic amount of argon flushed xanthine oxidase and spectra were collected every minute during the reaction using PerkinElmer Lambda Bio40 spectrophotometer. The log[ox/red] values of the enzyme were plotted against the log[ox/red] values of the reference dye and fitted using Nernst equation (Eq. 4.2).

$$E_h = E_m + \frac{2.303RT}{nF}\log\left[\frac{ox}{red}\right] \quad \text{(Eq. 4.2)}$$

RESULTS

6HDNO sequence similarity to bicovalent flavoproteins

All recently discovered bicovalent flavoproteins belong to the VAO flavoprotein family [49]. They are composed of two distinct domains typical for the family: the FAD-binding and the substrate-binding domains. Comparison of the elucidated crystal structures of bicovalent flavoproteins reveals that the two FAD-linking residues, histidine and cysteine, are located at structurally analogous positions. The histidine residue is located in the N-terminal part of the sequence, while the cysteine is found around 60 residues C-terminal from the linking histidine (Fig. 4.2A&B). Multiple sequence alignment analysis shows that the linking histidine is preceded by two strictly conserved glycines. A similar sequence characteristic has been observed for the monocovalent VAO-type proteins (Fig. 4.2A). The FAD-linking cysteine residue in bicovalent flavoproteins is not directly flanked by conserved residues. However, a remarkably glycine-rich region a few residues C-terminal from the cysteine can be identified in bicovalent flavoprotein sequences (7 conserved glycines in a stretch of 12 residues). Sequence analysis of VAO-type monocovalent flavoproteins reveals a much lower degree of conservation in this region (Fig. 4.2A). This suggests that the glycine-rich region, together with the conserved cysteine, can be used to identify bicovalent flavoprotein sequences. In case of the prototype monocovalent flavoprotein 6HDNO, FAD is covalently linked *via* His71 [25], which is the counterpart of the FAD-linking histidine in bicovalent flavoenzymes (Fig. 4.2A&B). However, in place of the cysteine forming the second covalent bond in bicovalent flavoproteins, 6HDNO has a histidine residue (His130) prohibiting the formation of a cysteinyl linkage

as the second covalent attachment (Fig. 4.2A&B). Knowing that (bi)covalent flavinylation is a self-catalytic process, we wanted to study whether it is feasible to create a bicovalent flavoprotein out of a monocovalent one.

As a first attempt to create a bicovalent 6HDNO, a single mutant was prepared in which only the linking cysteine was introduced: His130Cys 6HDNO. As we have experienced from our previous study on putrescine oxidase that introducing a covalent flavin-protein bond may require multiple mutations, we also decided to make a double mutant: His130Cys/Leu138His 6HDNO. The second mutation was inspired by the observation that the known bicovalent flavoproteins typically have a histidine residue within the glycine-rich region. This histidine is absent in wild-type 6HDNO (Fig. 4.2A). Recent studies have shown that a positive charge near the isoalloxazine moiety of the FAD cofactor is required for stabilization of the negative charge formed at the flavin ring system during the formation of the covalent bond [20,50]. The proposed mechanism of covalent flavinylation, studied in monocovalent flavoproteins [27,31,51], starts with a proton abstraction from the C8α-methyl group of the flavin and subsequent formation of the negative charge at the N1-C2=O locus of the isoalloxazine ring [20]. Possibly the histidine introduced in the double mutant could take up such role in facilitating the formation of the covalent Cys-FAD linkage. Inspection of the crystal structure of 6HDNO revealed that the positions of both targeted residues, His130 and Leu138, are compatible with their anticipated roles (Fig. 4.2B). The Cβ of His130 is only separated by 3.5 Å from the C6 of FAD while Leu is less than 3.5 Å away from the N1-C2=O locus of FAD.

(A)

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dbv29       86   VRSGGHCFEDF (52) GVCGGVGVGGHICGGGYG 166
TamL        57   VRSGGHCYEDF (52) GACPDVGAGGHILGGGYG 137
AknOx      108   VRSGGHCFEGF (52) GVCPQVGVGGHVLGGGYG 188
GilR        60   CRSGGHCGQDF (52) GACSAVGMGGLVAGGGYG 140
BBE         99   LRSGGHSYEGL (54) GWCPTVGTGGHISGGGFG 181
THCAs       82   TRSGGHDAEGM (54) GYCPTVGVGGHFSGGGYG 164
GOOX        90   AKGGGHSYGSY (52) GTCPAVGVGGHVLGGGYG 170
CHITO       89   AKSGGHSYTSL (52) GTCPGVGLGGHALHGGYG 169

Consensus        xxSGGHxxxxx      GxCxxVGxGGHxxGGGYG

6HDNO       65   VRSGGHNPNGY (53) GMHPKVGFCGLALNGGVG 146
CHO         64   PRGAMHGWTPL (57) NLPAPGVLSIGGALAVNA 150
ALDO        40   VLGSGHSFNEI (57) HISVAGSVATGTHGSGVG 127
ENCM        72   VRGGGHSMAGH (49) GVVSHTGLGGLVLGGGFG 152
CKX         99   FRGRGHSLMGQ (58) DYLYLTVGGTLSNAGISG 187

Consensus        xRGxGHxxxxx      xxxxxxxxxxxxxxxxxG
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(B)

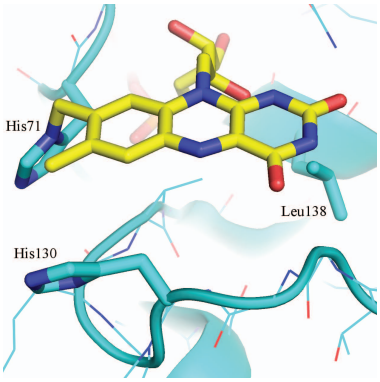

Figure 4.2. (A) Sequence alignment of monocovalent and bicovalent flavoproteins. Covalent flavinylation sites are in red, and the conserved histidine in bicovalent flavoproteins is in green. Consensus sequences for the mono- and bicovalent flavoprotein sequences are shown. Bicovalent flavoproteins: dbv29 - primary alcohol oxidase (UniProt nr. Q7WZ62); TamL - FAD-dependent tirandamycin oxidase TamL (UniProt nr. D3Y1I2); AknOx - aclacinomycin oxidoreductase (UniProt nr. Q0PCD7); GilR - GilR oxidoreductase (UniProt nr. Q7X2G7); BBE - reticuline oxidase (UniProt nr. P30986); THCAs - tetrahydrocannabinolic acid synthase (UniProt nr. Q8GTB6); GOOX - glucooligosaccharide oxidase (UniProt nr. Q6PW77); CHITO - chitooligosaccharide oxidase (UniProt nr. I1S2K2). Monocovalent flavoproteins: 6HDNO - 6-hydroxy-D-nicotine oxidase (UniProt nr. P08159); CHO - cholesterol oxidase (UniProt nr. Q7SID9); ALDO - alditol oxidase (UniProt nr. Q9ZBU1); EncM - EncM oxygenase (UniProt nr. Q9KHK2); CKX - cytokinin dehydrogenase 1 (UniProt nr. Q9T0N8). **(B)** View on the FAD cofactor as monocovalently bound in wild-type 6HDNO (PDB:2BVF). The cofactor is shown in yellow sticks while the protein part is in cyan. The linking histidine (His71) and the two residues targeted in this study (His130 and Leu138) are highlighted in sticks.

Flavin binding properties

Wild-type, His130Cys and His130Cys/Leu138His 6HDNO were constructed, expressed and purified as described in the Experimental procedures section. All proteins were found to be able to bind FAD as evidenced by the yellow appearance of the proteins. In addition, the measured 272/450 absorbance ratio ranged between 7-9, which agrees well with the value of 7.6 previously published for purified wild-type 6HDNO [44]. The purified proteins were tested for covalent attachment of the FAD cofactor by precipitation with 5% TCA. The obtained supernatant did not show any absorbance in the UV/Vis region, confirming the presence of a covalent bond between the cofactor and the protein in each 6HDNO variant. Also in-gel flavin fluorescence of all three purified proteins was detected upon SDS-PAGE, which further confirmed the ability of covalent flavinylation in all three proteins.

UV-visible absorption spectra were collected before and after unfolding of all three variants (Fig. 4.3). The spectrum of the native wild-type enzyme resembles the one previously published with absorbance maxima at 450, 355 and 272 nm and a shoulder at 475 nm [44]. The native and unfolded spectra of both, wild-type and His130Cys 6HDNO, do not differ significantly and they represent typical flavoprotein absorbance spectra of a histidyl-bound FAD. On the other hand, the spectra of the double mutant His130Cys/Leu138His differ markedly from the wild-type 6HDNO and are very unusual. In the native and unfolded state, the near UV absorbance peak at around 360 nm is not present (native state) or is much lower (unfolded state) when compared to wild-type and His130Cys 6HDNO, while a prominent peak in the native and unfolded state was observed at 436 and 439 nm, respectively. This suggests a significant change in the flavin binding mode. Intriguingly, very similar spectra have been previously reported for bicovalent flavoenzymes: the primary alcohol oxidase Dbv29 [52], chitooligosaccharide oxidase [53] and berberine bridge enzyme [35]. Altogether, the spectral features of the double mutant hint to an introduced Cys-FAD linkage.

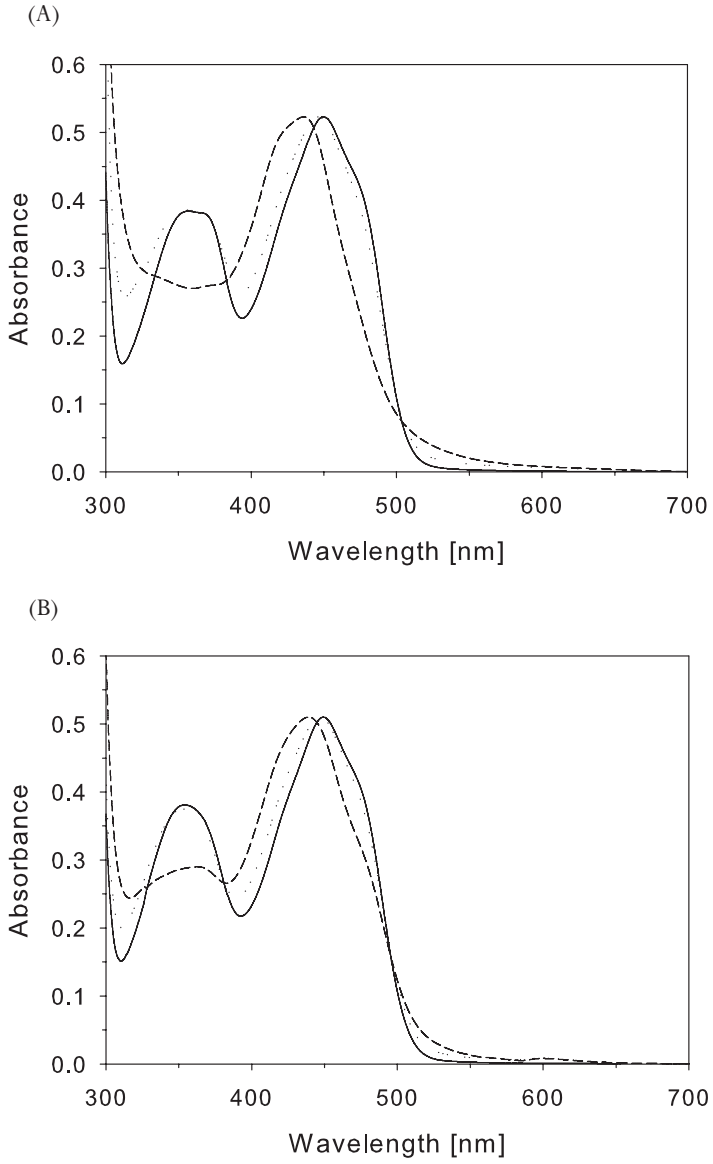

Figure 4.3. Absorption spectra of the native **(A)** and unfolded **(B)** 6HDNO variants. Solid lines represent data for wild-type, dotted lines for His130Cys and dashed lines for His130Cys/Leu138His enzyme. All spectra were normalized to a protein concentration of ~65 μM.

Wild-type 6HDNO binds FAD *via* an 8α-*N*1-histidyl bond, involving His71, as evidenced from the crystal structure [54]. From the absorbance spectral analysis, it seems that the His130Cys variant has the same type of covalent binding, while

His130Cys/Leu138His 6HDNO is able to form the second covalent attachment involving the introduced Cys130. In order to confirm this, the Ellman method was chosen to measure the number of free thiol groups in the peptides of unfolded 6HDNO variants. According to the amino acid sequence of wild-type 6HDNO, 6 cysteines should be determined. Both mutants have one cysteine introduced with the mutation, but in the double His130Cys/Leu138His mutant the additional cysteine cannot be detected, if it forms the covalent bond with the flavin. The results obtained show that in His130Cys/Leu138His 6HDNO, as well as in the wild-type, indeed only 6 cysteines can be detected (Table 4.1). However, in the single mutant only 6 cysteines were measured as well. This can be explained by the proximity of the thiol group of the introduced cysteine to the active site on the flavin isoalloxazine moiety and its high degree of solvent accessibility. This renders the cysteine prone to oxidation and would prevent it from being detected with Ellman's reagent.

Table 4.1. Characterization of 6HDNO variants. Cysteine content of the SDS-unfolded proteins was determined using the Ellman's method. Redox potentials were measured using a reference dye (specific dye and midpoint redox potential in brackets). Steady state kinetic parameters were determined using D-6-hydroxynicotine as a substrate. Melting temperatures were determined using the ThermoFAD and ThermoFluor methods for wild-type enzyme, yielding identical temperature for each enzyme variant, while for the double mutant only the ThermoFluor could be used.

	6HDNO		
	wild-type	**H130C**	**H130C/L138H**
Cysteines determined	6.0 ± 0.12	5.7 ± 0.14	5.6 ± 0.07
Redox potential			
dye [E_0, mV]	resorufin[-51]/ pyocyanin[-38]	resorufin[-51]/ pyocyanin[-38]	methylene blue[+11]
E_0 [mV]	-36.5±2.9	-35.0±1.4	+11.8
Kinetic parameters			
k_{cat} [s^{-1}]	20.7±0.8	6.7±0.4	0.89±0.2
K_M [μM]	22.1±1.9	52.6±1.6	99.9±21
K_i [μM]	840±82	-	-
k_{cat}/K_M [$mM^{-1}s^{-1}$]	943±83	126±4	9.0±1.3
Melting temperature [°C]	50	47	48

In order to confirm that the double mutant forms an additional covalent bond with the flavin, whereas the single mutant does not, the fluorescence spectra

of both native and unfolded proteins were measured. It is well known that flavin fluorescence is quenched in folded flavoproteins and it changes upon unfolding and exposing the flavin to the solvent. However, in proteins that have a flavin covalently bound to a cysteine, this specific amino acid–flavin linkage quenches flavin fluorescence [55,56]. It is apparent from the Figure 4.4 that the fluorescence increases significantly in wild-type and single mutant 6HDNO upon unfolding with SDS, while the change in the double mutant is much less pronounced. As reference a typical bicovalent flavoprotein, chitooligosaccharide oxidase, was also analysed concerning its fluorescence properties. This revealed very similar fluorescence features when compared to the double mutant.

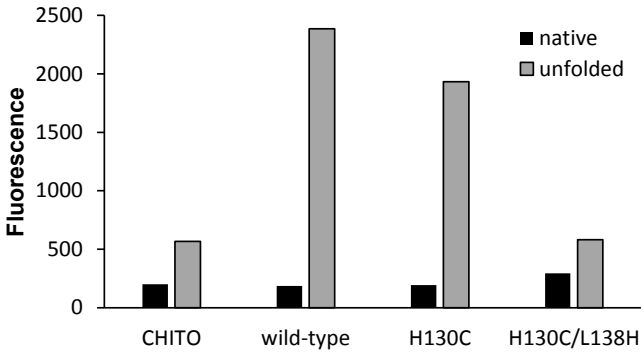

Figure 4.4. Fluorescence emission intensity at 522 nm of native and unfolded wild-type, His130Cys and His130Cys/Leu138His compared to bicovalent chitooligosaccharide oxidase. The values represent an average of at least 3 measurements with standard errors less tan 5%.

It has been shown that the flavin fluorescence of cysteinyl-FAD can be restored after cleavage of the covalent cofactor-protein linkage. According to Walker et al. [45], the Cys-flavin bond can be cleaved by oxidation with performic acid. After SDS-PAGE and spraying the gel with performic acid, clear fluorescent bands were observed for all three 6HDNO variants. This was not the case when the performic acid treatment step was omitted and in this case only wild-type and His130Cys 6HDNO bands were clearly fluorescent, while the His130Cys/Leu138His band was very faint in comparison. The quenching of in-gel fluorescence of the double mutant again confirms that the flavin-cysteine covalent bond was formed.

Redox potential determination

It is well known that modifications of the flavin cofactor as well as changes in the flavin microenvironment can influence the redox potential of the cofactor. For many flavoenzymes midpoint redox potentials have been determined and it was shown that (bi)covalent cofactor binding can dramatically increase the potential of a flavin cofactor [20]. The redox potentials measured for bicovalent flavoproteins are among the highest ever observed for flavoproteins, typically in the 0 to +200 mV range. In contrast, noncovalent flavoproteins typically exhibit redox potentials in the -400 to 0 mV range. In line with this, it has been observed that removal of a covalent flavin-protein bond by site-directed mutagenesis induces a drastic decrease in the redox potential. In fact, it has been postulated that the driving force for a protein to create a covalent flavin-protein bond is the increase of redox potential rendering an enzyme with a higher oxidative power [20]. Therefore, we set out to determine the redox properties of all three 6HDNO variants.

For that purpose, the xanthine/xanthine oxidase based method developed by Massey [48] was used. The enzymes were anaerobically reduced in the presence of a suitable reference dye (Table 4.1) and the absorbance data were collected. The ratios between oxidized and reduced flavin/dye were represented according to the Minnaert method [57]. From these plots the redox potentials could be calculated. For the His130Cys/Leu138His 6HDNO mutant, no radical intermediates could be observed during the reductions. The slope of the linear fit in the Nernst plot was 1.1, indicating a simultaneous 2-electron transfer for both flavoprotein and the dye, when they are in equilibrium. The midpoint redox potential obtained from the plot was 11.8 mV. The Nernst plots for the wild-type and His130Cys 6HDNO gave slopes of 0.46-0.62 for both reference dyes used. That suggests a 1-electron reduction of the flavin and 2-electron reduction of the dye. However, no flavin radical forms were observed for any of the two 6HDNO variants. One possible explanation could be a lack of equilibrium between the reduction of the cofactor and the reporter dye during the measurement, but this was ruled out by repeating the experiments at different xanthine oxidase concentrations, resulting in prolonged reductions lasting for up to 6 hours. Therefore, we conclude that 2-electron reduction in these two variants is kinetically inhibited, but the flavin radical form is not stabilized thermodynamically. This allows us to use the Nernst equation for the 2-electron reduction, resulting in midpoint redox potential

values of -37 and -35 mV for the wild-type and His130Cys 6HDNO, respectively. Compared to the His130Cys/Leu138His 6HDNO mutant, the redox potential of both variants is lower by almost 50 mV. These data are in agreement with the different FAD binding modes, as the 6HDNO variants: wild-type and His130Cys bind FAD monocovalently, while the double mutant is able to create an additional covalent attachment to the flavin.

Enzyme activity and stability

Table 4.1 shows the kinetic parameters measured for the three 6HDNO variants. All three enzyme variants display significant activity. The wild-type enzyme is by far the most active enzyme, showing a specificity constant 10 fold and 100 fold higher than the single and double mutant, respectively. The mutations affect both the k_{cat} and the K_M values, but the k_{cat} is influenced more significantly. This is in line with a preserved binding site for the substrate. Interestingly, the wild-type enzyme suffers from substrate inhibition while for the mutants no inhibition is observed for the tested substrate range. This correlates with the relatively high K_M values for the mutants, suggesting that the mutations result in a lower affinity for the substrate.

Beside an increase in redox potential, some other roles for (bi)covalent flavinylation have been postulated. One of them is to improve protein stability and integrity, because the removal of a covalent bond in a natural covalent flavoprotein can lead to the production of an incorrectly folded enzyme [58,59], thermal and structural instability [10,60] and complete deactivation or decrease in activity [10,61,62]. A variety of methods can be used to assess protein stability. Here we decided to use the ThermoFluor [47] and ThermoFAD [46] methods, which determine the apparent melting temperature of a protein. All three 6HDNO enzymes were found to have very similar melting temperatures: wild-type, 50 °C; His130Cys, 47 °C; and His130Cys/Leu138His, 48 °C. For the wild-type and His130Cys 6HDNO the melting temperatures did not depend on the employed method, ThermoFluor or ThermoFAD. For the double mutant only the ThermoFluor method could be used, because the generated flavin fluorescence signal was too low. This was most likely caused by the bicovalent attachment of the flavin, which alters the fluorescence properties of the flavin (see above). It is interesting to note that, although both mutants are somewhat less stable than the wild-type, the double mutant has a slightly increased stability compared to the single mutant.

DISCUSSION

The present study was designed to introduce a second covalent bond in 6HDNO and to reveal which features are important in the process of bicovalent flavinylation. Wild-type 6HDNO binds FAD in a monocovalent manner *via* an 8α-*N*1-histydyl linkage, similar to many other flavoproteins in the VAO-family. The FAD-binding domain of 6HDNO shows some sequence similarity to bicovalent flavoproteins, but lacks conservation of the linking cysteine and the flanking residues (Fig. 4.2A). The two major features missing in 6HDNO, namely the covalent cysteine and the conserved histidine, were introduced, resulting in two mutants: His130Cys and His130Cys/Leu138His. The analysis of the type of covalent binding in all three 6HDNO variants showed that a single mutation of the histidine to the cysteine is not sufficient for the second bond to be formed between FAD and the enzyme. Only when a histidine is introduced in the double mutant His130Cys/Leu138His 6HDNO, does bicovalent flavinylation take place. Little is known about the mechanistic aspects of bicovalent flavinylation, except that it has been shown that both bonds are formed independently from each other [10,18-20,35]. The results of this study indicate that the second covalent bond, the Cys-FAD linkage, is only formed when a histidine is present near the N1-C2=O locus of the isoalloxazine ring. This suggests that there is a need for a positive charge to stabilize the negative charge that develops on the isoalloxazine moiety of the flavin cofactor during the Cys-FAD flavinylation reaction. The results also suggest that the histidyl-FAD bond is formed more easily. This is in line with the observation that the majority of covalent flavoproteins are histidyl-FAD containing enzymes. It also hints to an evolutionary order of formation of the two covalent FAD-protein linkages in VAO-type proteins: first the histidyl-FAD linkage was established, followed by the introduction of the cysteinyl-FAD linkage. Such an order of events is also seen when performing a multiple sequence alignment of VAO-type proteins. The bicovalent flavoproteins form a distinct clade that seem to originate from a common monocovalent flavoprotein [49].

The introduced histidine in 6HDNO, His138, is at a position that is typically occupied by a histidine in natural bicovalent flavoproteins. While we demonstrate that the introduced histidine in 6HDNO is beneficial for the formation of a second covalent bond between flavin and 6HDNO, research of Wallner et al. [63] showed that the corresponding histidine in the bicovalent flavoprotein berberine bridge

enzyme is not an absolute requirement for bicovalent flavinylation. When His174 in berberine bridge enzyme is mutated to an alanine, the bicovalent linkage is still formed. However, the Cys-flavin bond in the His174Ala berberine bridge enzyme mutant [18,35] is lost during anaerobic photoreduction and only slowly reformed after addition of oxygen. His174 in berberine bridge enzyme interacts with the C2' hydroxyl group of the ribityl side chain of the flavin that in turn interacts with the N1-C2=O locus of the isoalloxazine ring system. This indicates the importance of the histidine in stabilization of the reduced form of the cofactor, where the negative charge is formed in the N1-C2=O locus. However, in addition to the histidine, another amino acid, Tyr456, takes part in hydrogen bond network in berberine bridge enzyme and which may be sufficient for mediating formation of the Cys-FAD linkage in the absence of His174. In 6HDNO, the corresponding tyrosine is not present and this might be the reason, why the introduced histidine is essential for the Cys-flavin bond formation.

The significance of (bi)covalent flavin binding has been demonstrated many times in mutagenesis studies of mono- and bicovalent flavoenzymes, where removal of a covalent linkage resulted in a decrease of the midpoint redox potential [20]. It indicates that one of the functions of bicovalent flavin binding is to increase the oxidative power of an enzyme. This study produced results which corroborate this finding, because the redox potential of bicovalent 6HDNO increased compared to the two other monocovalent enzyme variants. However, the natural bicovalent flavoenzymes have somewhat higher midpoint redox potentials (glucooligosaccharide oxidase +126 mV [19], chitooligosaccharide oxidase +131 mV [10], berberine bridge enzyme +132 mV [18]) than the engineered bicovalent 6HDNO (+12 mV). This is not surprising, because wild-type 6HDNO also shows a relatively low midpoint redox potential (-37 mV) when compared to other monocovalent VAO-type flavoproteins (*p*-cresolmethylhydroxylase +84 mV; vanillyl-alcohol oxidase +50 mV; cytokinin dehydrogenase +8 mV; eugenol oxidase -2 mV) [20]. Mutations leading to a removal of the Cys-FAD bond in bicovalent flavoenzymes usually cause a decrease in redox potential of about 60-80 mV. Therefore, the difference of almost 50 mV between monocovalent wild-type and bicovalent mutant 6HDNO is in the expected range. Most probably, there are also other amino acids in the flavin-binding pocket of bicovalent flavoproteins which influence the redox potential *via* non-covalent interactions. In 6HDNO these interactions might be missing.

When considering enzyme functioning, a major drawback of the mutations introduced in 6HDNO is that they lead to a decrease in activity of the enzyme. A possible explanation for this might be the location of the mutations. The His130 is substituted by a cysteine while it is located next to the C6 isoalloxazine ring locus, which is in direct proximity of the hydride-accepting N5 atom of the FAD cofactor. The introduced cysteine also forms a part of the substrate binding pocket [54]. Therefore the amino acid replacement may cause perturbations in the 6-hydroxy-D-nicotine binding and subsequent oxidation. The histidine that was introduced in place of leucine 138 is located on the other side of the isoalloxazine ring but it mediates the formation of a Cys-FAD covalent bond. The newly formed covalent bond, although it increases the redox potential, may induce an altered positioning of the isoalloxazine moiety in the active site. This in turn may hamper proper positioning of the substrate for optimal hydride transfer. Additionally to that, the increased redox potential may not be optimal for catalysis. However, both mutations do not cause significant perturbations of the protein structure, as all three variants have very similar thermostability, display similar K_M values and are still active. Future detailed (pre-steady state) kinetic studies should shed more light on these aspects of 6HDNO-mediated catalysis. It is worth noting that Koetter et al. [54] showed that the active centre in 6HDNO is surprisingly large when considering 6-hydroxy-D-nicotine as substrate. They suggested that the real physiological 6HDNO substrate may not have been discovered yet. If this is true, it would be interesting to see if an increased redox potential in 6HDNO would lead to an increase in catalytic activity with the natural substrate.

This study provides new insights into the self-catalytic mechanism of bicovalent flavinylation of flavoproteins. We used monocovalent 6HDNO as a prototype flavoprotein and were able to introduce an additional second covalent flavin-protein linkage. Our results indicate that a specific flavin microenvironment is required for the formation of a Cys-FAD linkage, which should include a positive charge to stabilize a negatively charged flavin flavinylation intermediate (His138 in 6HDNO). In most known natural bicovalent flavoproteins a histidine is also found at the corresponding position. Our study also shows that it is feasible *via* protein engineering to turn a monocovalent flavoprotein into a bicovalent flavoprotein.

REFERENCES

[1] O. Warburg, W. Christian, Ein zweites sauerstoffübertragendes Ferment und sein Absorptionsspektrum, Naturwissenschaften. 20 (1932) 688-688.

[2] S. Ghisla, V. Massey, Mechanisms of flavoprotein-catalyzed reactions, Eur. J. Biochem. 181 (1989) 1-17.

[3] P. Macheroux, B. Kappes, S.E. Ealick, Flavogenomics – a genomic and structural view of flavin-dependent proteins, FEBS J. 278 (2011) 2625-2634.

[4] M. Mewies, W.S. McIntire, N.S. Scrutton, Covalent attachment of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to enzymes: the current state of affairs, Protein Sci. 7 (1998) 7-20.

[5] Y. Nakayama, M. Yasui, K. Sugahara, M. Hayashi, T. Unemoto, Covalently bound flavin in the NqrB and NqrC subunits of Na^+-translocating NADH-quinone reductase from *Vibrio alginolyticus*, FEBS Lett. 474 (2000) 165-168.

[6] Y.V. Bertsova, M.S. Fadeeva, V.A. Kostyrko, M.V. Serebryakova, A.A. Baykov, A.V. Bogachev, Alternative Pyrimidine Biosynthesis Protein ApbE Is a Flavin Transferase Catalyzing Covalent Attachment of FMN to a Threonine Residue in Bacterial Flavoproteins, J. Biol. Chem. 288 (2013) 14276-14286.

[7] K. Podzelinska, R. Latimer, A. Bhattacharya, L.C. Vining, D.L. Zechel, Z. Jia, Chloramphenicol biosynthesis: the structure of CmlS, a flavin-dependent halogenase showing a covalent flavin-aspartate bond, J. Mol. Biol. 397 (2010) 316-331.

[8] C.H. Huang, W.L. Lai, M.H. Lee, et al., Crystal structure of glucooligosaccharide oxidase from *Acremonium strictum*, J. Biol. Chem. 280 (2005) 38831-38838.

[9] A. Winkler, F. Hartner, T.M. Kutchan, A. Glieder, P. Macheroux, Biochemical evidence that berberine bridge enzyme belongs to a novel family of flavoproteins containing a bi-covalently attached FAD cofactor, J. Biol. Chem. 281 (2006) 21276-21285.

[10] D. Heuts, R. Winter, G. Damsma, D. Janssen, M. Fraaije, The role of double covalent flavin binding in chito-oligosaccharide oxidase from *Fusarium graminearum*, Biochem. J. 413 (2008) 175-183.

[11] M.K. Kharel, P. Pahari, H. Lian, J. and Rohr, GilR, an unusual lactone-forming enzyme involved in gilvocarcin biosynthesis, ChemBioChem. 10 (2009) 1305-1308.

[12] X. Mo, H. Huang, J. Ma, et al., Characterization of TrdL as a 10-Hydroxy Dehydrogenase and Generation of New Analogues from a Tirandamycin Biosynthetic Pathway, Org. Lett. (2011).

[13] T. Rand, K.B. Qvist, C.P. Walter, C.H. Poulsen, Characterization of the flavin association in hexose oxidase from *Chondrus crispus*, FEBS Journal. 273 (2006) 2693-2703.

[14] M. Sosio, S. Stinchi, F. Beltrametti, A. Lazzarini, S. Donadio, The gene cluster for the biosynthesis of the glycopeptide antibiotic A40926 by Nonomuraea species, Chem. Biol. 10 (2003) 541-549.

[15] Y. Shoyama, T. Tamada, K. Kurihara, et al., Structure and Function of Δ1-Tetrahydrocannabinolic Acid (THCA) Synthase, the EnzymeControlling the Psychoactivity of *Cannabis sativa*, J. Mol. Biol. (2012).

[16] I. Alexeev, A. Sultana, P. Mäntsälä, J. Niemi, G. Schneider, Aclacinomycin oxidoreductase (AknOx) from the biosynthetic pathway of the antibiotic aclacinomycin is an unusual flavoenzyme with a dual active site, Proceedings of the National Academy of Sciences. 104 (2007) 6170.

[17] M.W. Fraaije, van den Heuvel, Robert HH, W.J. van Berkel, A. Mattevi, Covalent flavinylation is essential for efficient redox catalysis in vanillyl-alcohol oxidase, J. Biol. Chem. 274 (1999) 35514-35520.

[18] A. Winkler, T.M. Kutchan, P. Macheroux, 6-*S*-cysteinylation of bi-covalently attached FAD in berberine bridge enzyme tunes the redox potential for optimal activity, J. Biol. Chem. 282 (2007) 24437-24443.

[19] C.H. Huang, A. Winkler, C.L. Chen, et al., Functional roles of the 6-*S*-cysteinyl, 8α-*N*1-histidyl FAD in glucooligosaccharide oxidase from *Acremonium strictum*, J. Biol. Chem. 283 (2008) 30990-30996.

[20] D.P. Heuts, N.S. Scrutton, W.S. McIntire, M.W. Fraaije, What's in a covalent bond? On the role and formation of covalently bound flavin cofactors, FEBS J. 276 (2009) 3405-3427.

[21] M.W. Fraaije, W.J.H. van Berkel, J.A.E. Benen, J. Visser, A. Mattevi, A novel oxidoreductase family sharing a conserved FAD-binding domain, Trends Biochem. Sci. 23 (1998) 206-207.

[22] W.P. Dijkman, G. de Gonzalo, A. Mattevi, M.W. Fraaije, Flavoprotein oxidases: classification and applications, Appl. Microbiol. Biotechnol. 97 (2013) 5177-5188.

[23] K. Decker, H. Bleeg, Induction and purification of stereospecific nicotine oxidizing enzymes from *Arthrobacter oxidans*, Biochimica et Biophysica Acta (BBA)-Enzymology and Biological Oxidation. 105 (1965) 313-324.

[24] R. Brandsch, V. Bichler, H. Nagursky, Covalent flavinylation of 6-hydroxy-D-nicotine oxidase analyzed by partial deletions of the gene, European Journal of Biochemistry. 165 (1987) 559-564.

[25] H. Möhler, M. Brühmüller, K. Decker, Covalently Bound Flavin in D-6-Hydroxynicotine Oxidase from *Arthrobacter oxidans*, European Journal of Biochemistry. 29 (1972) 152-155.

[26] H. Nagursky, V. Bichler, R. Brandsch, Phosphoenolpyruvate-dependent flavinylation of 6-hydroxy-D-nicotine oxidase, European Journal of Biochemistry. 177 (1988) 319-325.

[27] R. Brandsch, V. Bichler, Autoflavinylation of apo6-hydroxy-D-nicotine oxidase, J. Biol. Chem. 266 (1991) 19056-19062.

[28] J. Kim, J.H. Fuller, V. Kuusk, et al., The cytochrome subunit is necessary for covalent FAD attachment to the flavoprotein subunit of p-cresol methylhydroxylase, J. Biol. Chem. 270 (1995) 31202-31209.

[29] K.M. Robinson, B.D. Lemire, Covalent attachment of FAD to the yeast succinate dehydrogenase flavoprotein requires import into mitochondria, presequence removal, and folding, J. Biol. Chem. 271 (1996) 4055-4060.

[30] P. Trickey, M.A. Wagner, M.S. Jorns, F.S. Mathews, Monomeric sarcosine oxidase: structure of a covalently flavinylated amine oxidizing enzyme, Structure. 7 (1999) 331-345.

[31] J. Jin, H. Mazon, R.H. van den Heuvel, A.J. Heck, D.B. Janssen, M.W. Fraaije, Covalent flavinylation of vanillyl-alcohol oxidase is an autocatalytic process, FEBS J. 275 (2008) 5191-5200.

[32] B. Barquera, C.C. Häse, R.B. Gennis, Expression and mutagenesis of the NqrC subunit of the NQR respiratory Na^+ pump from *Vibrio cholerae* with covalently attached FMN, FEBS Lett. 492 (2001) 45-49.

[33] H. Hao, O. Khalimonchuk, M. Schraders, et al., SDH5, a gene required for flavination of succinate dehydrogenase, is mutated in paraganglioma, Science. 325 (2009) 1139-1142.

[34] M.B. McNeil, J.S. Clulow, N.M. Wilf, G.P. Salmond, P.C. Fineran, SdhE is a conserved protein required for flavinylation of succinate dehydrogenase in bacteria, J. Biol. Chem. 287 (2012) 18418-18428.

[35] A. Winkler, K. Motz, S. Riedl, M. Puhl, P. Macheroux, K. Gruber, Structural and mechanistic studies reveal the functional role of bicovalent flavinylation in berberine bridge enzyme, J. Biol. Chem. 284 (2009) 19993-20001.

[36] E.W. van Hellemond, H. Mazon, A.J. Heck, et al., ADP competes with FAD binding in putrescine oxidase, J. Biol. Chem. 283 (2008) 28259-28264.

[37] M.M. Kopacz, S. Rovida, E. van Duijn, M.W. Fraaije, A. Mattevi, Structure-based redesign of cofactor binding in putrescine oxidase, Biochemistry. 50 (2011) 4209-4217.

[38] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1959) 70-77.

[39] A.F. Habeeb, Reaction of protein sulfhydryl groups with Ellman's reagent, Methods Enzymol. 25 (1972) 457-464.

[40] M. Larkin, G. Blackshields, N. Brown, et al., Clustal W and Clustal X version 2.0, Bioinformatics. 23 (2007) 2947-2948.

[41] M. Goujon, H. McWilliam, W. Li, et al., A new bioinformatics analysis tools framework at EMBL–EBI, Nucleic Acids Res. 38 (2010) W695-W699.

[42] R. Fraczkiewicz, W. Braun, Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules, Journal of Computational Chemistry. 19 (1998) 319-333.

[43] http://www.expasy.ch/sprot/

[44] M. Brühmüller, H. Möhler, K. Decker, Covalently Bound Flavin in D-6-Hydroxynicotine Oxidase from *Arthrobacter oxidans*, European Journal of Biochemistry. 29 (1972) 143-151.

[45] W.H. Walker, E.B. Kearney, R.L. Seng, T.P. Singer, The covalently-bound flavin of hepatic monoamine oxidase. 2. Identification and properties of cysteinyl riboflavin, Eur. J. Biochem. 24 (1971) 328-331.

[46] F. Forneris, R. Orru, D. Bonivento, L.R. Chiarelli, A. Mattevi, ThermoFAD, a Thermofluor®-adapted flavin ad hoc detection system for protein folding and ligand binding, FEBS Journal. 276 (2009) 2833-2840.

[47] M.W. Pantoliano, E.C. Petrella, J.D. Kwasnoski, et al., High-density miniaturized thermal shift assays as a general strategy for drug discovery, Journal of biomolecular screening. 6 (2001) 429-440.

[48] V. Massey, A simple method for the determination of redox potentials, Flavins and flavoproteins. (1991) 59-66.

[49] N.G. Leferink, D.P. Heuts, M.W. Fraaije, W.J. van Berkel, The growing VAO flavoprotein family, Arch. Biochem. Biophys. 474 (2008) 292-301.

[50] M.W. Fraaije, A. Mattevi, Flavoenzymes: diverse catalysts with recurrent features, Trends Biochem. Sci. 25 (2000) 126-132.

[51] A. Hassan-Abdallah, R.C. Bruckner, G. Zhao, M.S. Jorns, Biosynthesis of covalently bound flavin: isolation and in vitro flavinylation of the monomeric sarcosine oxidase apoprotein, Biochemistry. 44 (2005) 6452-6462.

[52] Y.S. Li, J.Y. Ho, C.C. Huang, et al., A unique flavin mononucleotide-linked primary alcohol oxidase for glycopeptide A40926 maturation, J. Am. Chem. Soc. 129 (2007) 13384-13385.

[53] D.P. Heuts, D.B. Janssen, M.W. Fraaije, Changing the substrate specificity of a chitooligosaccharide oxidase from *Fusarium graminearum* by model-inspired site-directed mutagenesis, FEBS Lett. 581 (2007) 4905-4909.

[54] J.W. Koetter, G.E. Schulz, Crystal structure of 6-hydroxy-D-nicotine oxidase from *Arthrobacter nicotinovorans*, J. Mol. Biol. 352 (2005) 418-428.

[55] W.C. Kenney, T.P. Singer, Evidence for a thioether linkage between the flavin and polypeptide chain of Chromatium cytochrome c 552. J. Biol. Chem. 252 (1977) 4767-4772.

[56] M.C. Falk, D.B. McCormick, Synthetic flavinyl peptides related to the active site of mitochondrial monoamine oxidase. II. Fluorescence properties, Biochemistry (N. Y.). 15 (1976) 646-653.

[57] K. Minnaert, Measurement of the equilibrium constant of the reaction between cytochrome c and cytochrome a, Biochim. Biophys. Acta. 110 (1965) 42-56.

[58] D.P.H.M. Heuts, E.W. van Hellemond, D.B. Janssen, M.W. Fraaije, Discovery, characterization, and kinetic analysis of an alditol oxidase from *Streptomyces coelicolor*, J. Biol. Chem. 282 (2007) 20283-20291.

[59] R.K. Nandigama, D.E. Edmondson, Influence of FAD structure on its binding and activity with the C406A mutant of recombinant human liver monoamine oxidase A, J. Biol. Chem. 275 (2000) 20527-20532.

[60] L. Caldinelli, S. Iametti, A. Barbiroli, et al., Dissecting the structural determinants of the stability of cholesterol oxidase containing covalently bound flavin, J. Biol. Chem. 280 (2005) 22572-22581.

[61] M. Eschenbrenner, L.J. Chlumsky, P. Khanna, F. Strasser, M.S. Jorns, Organization of the multiple coenzymes and subunits and role of the covalent flavin link in the complex heterotetrameric sarcosine oxidase, Biochemistry (N.Y.). 40 (2001) 5352-5367.

[62] G. Cecchini, Function and Structure of Complex II of the Respiratory Chain, Annu. Rev. Biochem. 72 (2003) 77-109.

[63] S. Wallner, A. Winkler, S. Riedl, et al., Catalytic and structural role of a conserved active site histidine in berberine bridge enzyme, Biochemistry (N.Y.). (2012).

CHAPTER 5

SELECTIVE DEUTERATION OF FLAVIN COFACTORS

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ABSTRACT

Riboflavin (vitamin B2) is a precursor for naturally occurring flavins, namely flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). These two cofactors are used by a number of enzymes, called flavoenzymes. In order to analyse flavin function, covalent flavin binding process and to create enzymes with new catalytic properties chemically modified flavins are often used. One of such modifications is isotopic labelling, in particular deuteration. A method for specific deuteration of FMN at C8 methyl group is presented. Furthermore, FMN is enzymatically converted to riboflavin.

INTRODUCTION

The (bio)chemical properties of flavin cofactors originate from their redox-active isoalloxazine moiety, which can catalyse one- or two-electron transfer reactions and dioxygen activation [1,2]. In most cases flavin cofactors are bound tightly, but non-covalently, by the protein. However, covalent bond with the flavin has been found in approximately 10% of flavoproteins and in the vast majority the attachment is formed with the isoalloxazine ring moiety [3]. Provided that the original flavin cofactor can be removed by (local) unfolding of the flavoprotein with non-covalently bound flavin, most of so-created apo proteins will accept chemically-modified flavins as an alternative cofactor [4]. Creating flavoproteins that contain a modified isoalloxazine moiety can be useful in analysis of reaction mechanisms catalysed by the corresponding flavoenzymes. When the modification affects the attachment point of covalent binding to the flavin, effects on covalent flavinylation can be also probed. Furthermore, artificial flavoenzymes with new catalytic properties can be obtained by combining flavin derivatives with different protein scaffolds [5,6] (and Chapter 2). A large number of flavin derivatives with different substituents at various positions in the isoalloxazine ring system have been prepared. More information on their use in elucidating the mechanistic details of flavoprotein function and covalent flavinylation can be found in recent reviews [7–9].

Flavins are composed of a heterocyclic 7,8-dimethylisoalloxazine ring system (Fig. 5.1) and the difference between them concerns the modifications and substitutions in tricyclic moiety. The most known flavin molecule is riboflavin (vitamin B_2), a precursor for natural flavin cofactors. Riboflavin is also often added to food as a colouring agent. The most common flavin cofactors present in proteins are flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which differ in the group present at the N10 of the flavin. The substituent at the N10 is responsible for proper flavin binding to the protein, while the catalytically active part is located at isoalloxazine, typically at the C4a-N5 site. The activity of the flavin is modulated by the interactions with the amino acids of the hosting protein, but it can be also changed by isoalloxazine modifications. Substitutions at the C8 methyl group are of special interest, because this group has a functional role in many enzymes. For example, it is suggested to take part in electron-transfer processes in reductase domains and in photolyases [10,11]. It is also the group that forms the most common type of covalent flavin-protein bond linkage (see

Chapters 1, 3 and 4). Therefore, many different C8-flavin analogues were tested for the correlation between enzyme activity and proper covalent bond formation. For example, monoamine oxidases A and B were active only when they were able to form the covalent bond with the substituted flavin [12].

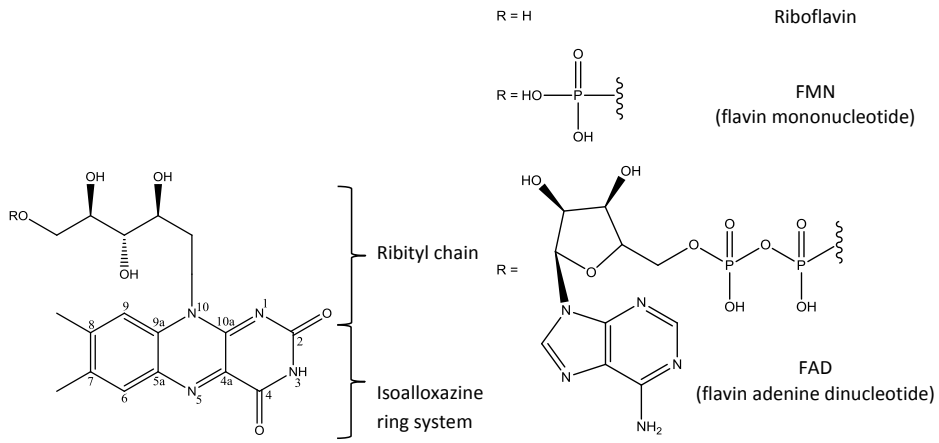


Figure 5.1. The structure of naturally occurring flavin cofactors.

The examples presented above demonstrate the power of using chemically-modified flavin cofactors for the elucidation of enzyme properties or for tuning enzyme reactivity. On the other hand, more subtly modified flavins, in which isotopes are incorporated, can also be valuable probes to study flavoproteins. For example, by using isotopically labelled flavins, NMR studies on flavoproteins are facilitated [13–23]. The use of isotopes is also a very common approach to unveil or study specific kinetic events in an enzyme reaction mechanism. Often, hydrogen-deuterium substitution is used, which is expected to slow down the reaction rate when C-H bond breaking is involved, causing a so called kinetic isotope effect. Interestingly, it has been proposed that deprotonation of the C8 methyl group is the first and possibly rate limiting step in the formation of a flavin-protein covalent bond [24–28]. Therefore, it would be worth to study the effect of using C8 methyl-deuterated flavin on the process of covalent flavinylation. In this Chapter, the preliminary results for synthesis of C8 methyl deuterated riboflavin and FMN are presented.

EXPERIMENTAL PROCEDURES

Chemicals

D_2O and NaOD were from Sigma-Aldrich, DCl from Acros Organics, Antarctic Phosphatase from New England BioLabs and TLC Silica gel 60/Kieselguhr F_{254} plates were from Merck.

Deuteration reaction

For the deuteration reaction of riboflavin, 100 mg riboflavin was resuspended in 900 µl D_2O and the pD adjusted with 5% NaOD until riboflavin was completely dissolved. Then the solution was heated at 93°C for approx. 42 h. A sample incubated at room temperature was used as a control.

For the deuteration reaction of FMN, the method of Bullock and Jardetzky was used [29]. 100 mg FMN was dissolved in 1 ml of 200 mM KPi prepared in D_2O and pD adjusted with DCl to 7.5. Then the solution was incubated at 90°C and small samples were collected at different time points for NMR and ESI-MS analysis.

NMR analysis

For monitoring the deuteration process, NMR was used. ^{1}H-NMR spectra of the flavins were recorded on a Varian AMX400 (400 MHz) in the solution used for deuteration reaction.

ESI-MS analysis

For electrospray ionization mass spectrometry experiments, samples of the deuteration reaction were prepared in 50% methanol with 10% acetic acid (riboflavin) or with 5% formic acid (FMN). The mass spectra were recorded on an ESI-MS (LCQ Fleet Ion Trap Thermo Fisher Scientific) in a positive mode.

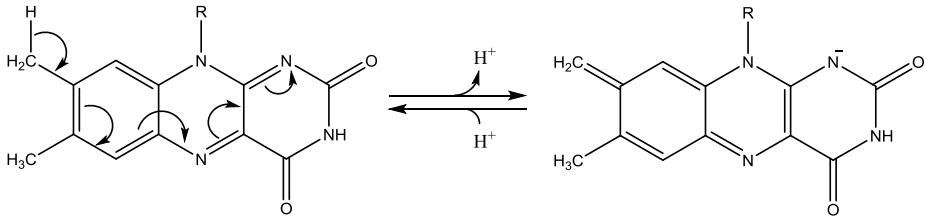
FMN dephosphorylation

25 mg/ml FMN was incubated with 5 U/µl Antarctic Phosphatase at 37°C and the reaction was monitored with TLC using 5% Na_2HPO_4 in 10% methanol as resolving system. The plates were inspected visually for the flavins.

RESULTS AND DISCUSSION

Deuteration of riboflavin

It was shown already in the 1960s that heating FMN at 90-95°C in a buffered D_2O solution leads to a selective proton exchange between the solvent and the C8 methyl group [29]. The authors proposed that the exchange takes place *via* an intermediate with a highly delocalized negative charge in the pyrimidine ring. This iminoquinone methide intermediate was later proposed to be an intermediate in the reaction of covalent bond formation between the C8 methyl group of FAD and the protein amino acid (Fig. 5.2) [30]. To investigate the process of covalent flavinylation in more detail, we wanted to synthetize a flavin compound deuterated at C8 methyl group.



iminoquinone methide

Figure 5.2. Formation of the flavin iminoquinone methide by deprotonation at the C8 methyl position.

For that purpose, we chose to prepare a riboflavin deuterated at C8 methyl position, although the reaction had only been shown to work for FMN [29]. Riboflavin was chosen because a riboflavin-auxotrophic *E. coli* strain, namely BSV11, carries mutations that render it permeable to this type of flavin [31]. Therefore, the modified riboflavin can be used *in vivo* to express the flavoprotein in its presence.

The low solubility of riboflavin in water was expected to inhibit the exchange of protons with the solvent. To overcome this problem, the riboflavin was solubilized by the addition of NaOD. First, a riboflavin reference solution was analysed with ^{1}H-NMR using D_2O/KOH as a solvent. Based on the literature [29,32] two signals of equal height were assigned to the two methyl groups of the isoalloxazine ring of the riboflavin: 2.01 ppm for the C7 and 2.12 ppm for the C8 methyl group. After the deuteration reaction, the signal corresponding to the C8 methyl group was significantly lower as compared to the signal of the C7 methyl group. However, C8 protons were still clearly present, indicating that the deuteration was not complete. Overall, the spectra of the deuterated compound were of rather low quality when compared to the non-deuterated riboflavin. The explanation for this came from ESI-MS analysis. It was clear that some deuterated riboflavin was formed, because the distribution of ion signals changed in favour of higher mass ions (ions at m/z 377 and 388 were virtually equal), but the conversion was not complete, as the substitution of three protons with deuteriums would lead to an increase of m/z by 3. Furthermore, the riboflavin ion signals were not much higher than the background signal and the same was found for the control incubated at room temperature. This indicates that the high pD used to solubilize riboflavin caused its degradation even at room temperature.

Deuteration of FMN

Because the problem with solubility of riboflavin rendered the deuteration reaction very inefficient, we decided to use FMN for the deuteration reaction. Deuterated FMN can subsequently be converted to riboflavin (Fig. 5.3). FMN, as a soluble compound, could be used at more neutral pH for the deuteration reaction. As can be seen from the ^{1}H-NMR spectra (Fig. 5.4), the signal corresponding to the C8 methyl group [29,32] decreased in the FMN sample incubated at 90°C and after 20 hours it disappeared completely. This was not observed with the sample incubated at room temperature. Here, both methyl groups gave a similar NMR signal, indicating that the H-D exchange reaction took place only at relatively high temperature. The number of deuteriums introduced was verified by ESI-MS (Fig. 5.5). The FMN mass in the heated sample increased indeed by 3 Da within 20 hours. The deuteration reaction at room temperature only yielded a slight increase in higher mass ions. The analysis with both methods, NMR and ESI-MS,

confirmed that the deuterium-hydrogen exchange reaction was complete and selective for only the C8 methyl group of FMN.

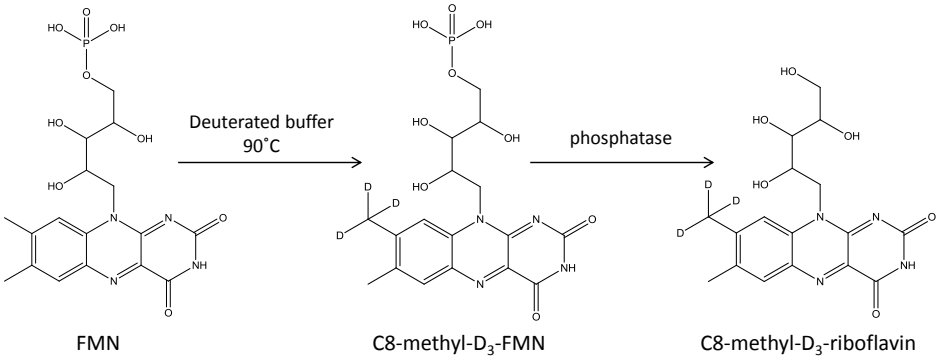


Figure 5.3. Reaction scheme for the synthesis of deuterated riboflavin, starting from FMN.

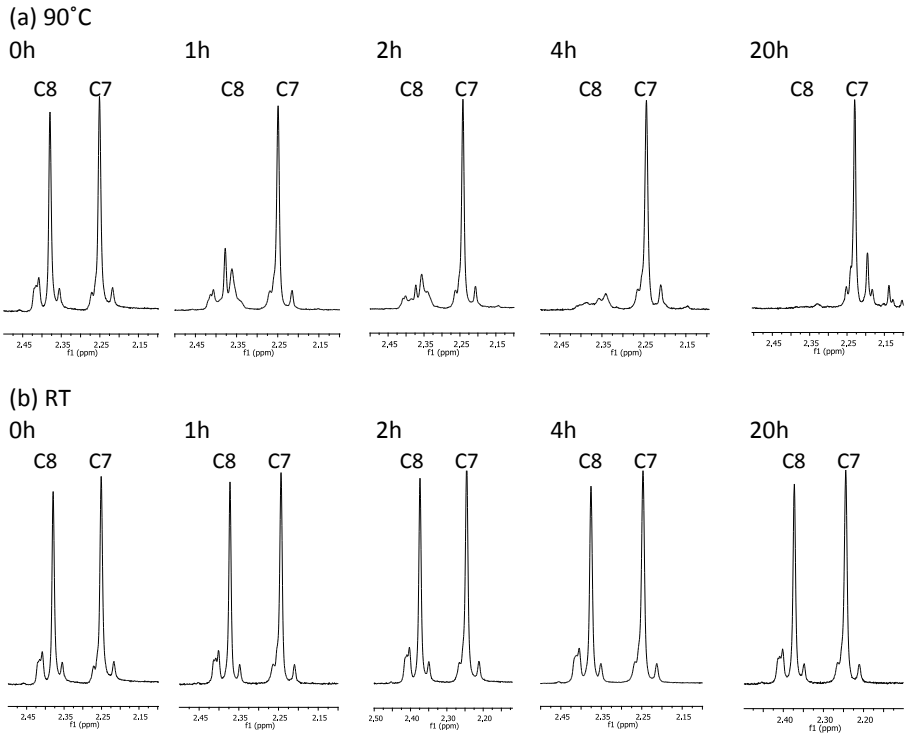


Figure 5.4. The methyl region of the ^{1}H-NMR spectra of FMN during the deuteration reaction at (a) 90˚C and (b) room temperature (RT). Samples were taken after 0, 1, 2, 4 and 20 hours. C8 and C7 correspond to the C8- and the C7-methyl hydrogens of the FMN.

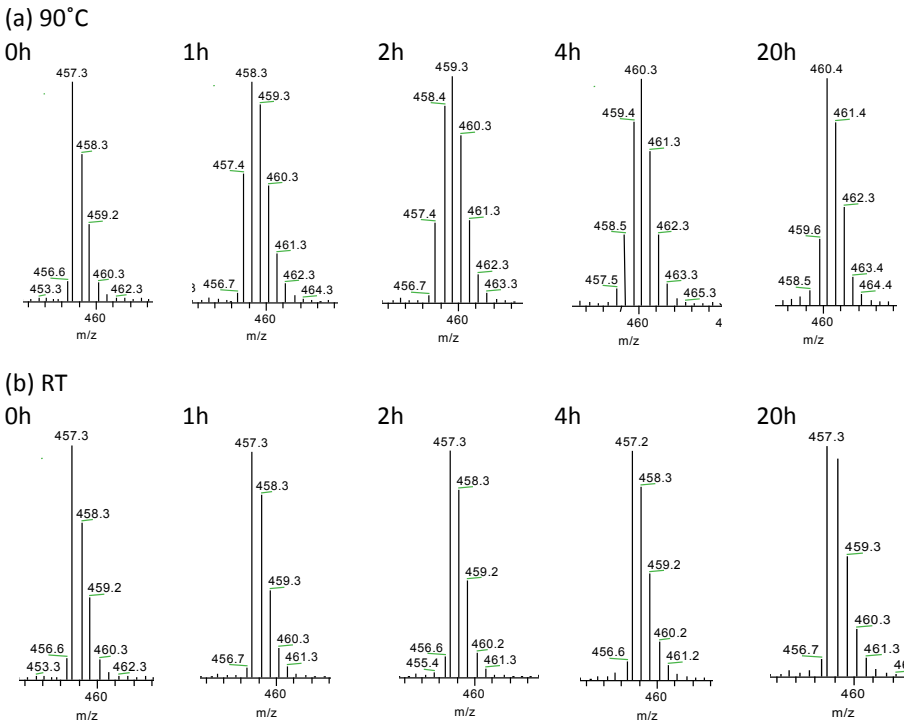


Figure 5.5. The FMN region of the electrospray (+) mass spectra of FMN at 0, 1, 2, 4 and 20 hours during the deuteration reaction at (a) 90˚C and (b) room temperature (RT).

FMN conversion to riboflavin

In order to dephosphorylate FMN, Antarctic Phosphatase was used and the reaction was monitored with thin layer chromatography (TLC). Again, because of the low solubility of riboflavin, it was difficult to find resolving conditions for both, riboflavin and FMN. For that reason, TLC was used only to estimate the progress of the conversion. After 3 days, the sample of the reaction looked similarly on TLC plate when compared to a riboflavin sample. Therefore, the hydrolysed sample was analysed by ESI-MS. This confirmed full conversion as only ions of m/z 377 belonging to riboflavin were found.

CONCLUSIONS

The results presented here show that specific deuteration of the C8 methyl group of FMN is feasible. We also showed that the deuterated FMN can be converted enzymatically to riboflavin. Such selectively-deuterated riboflavin is an interesting compound for investigating covalent flavinylation in proteins. Riboflavin can be used in the cells as FAD cofactor precursor. By using deuterated riboflavin in combination with riboflavin auxotrophic strains, such as *E. coli* BSV11 [31], it will be possible to study effects of deuteration on the efficiency of covalent flavinylation. Alternatively, deuterated riboflavin or FMN can be converted by using FAD synthetase into the FAD form. This would allow investigating the effects of C8 deuteration on the kinetics of covalent flavinylation.

REFERENCES

[1] L. Fruk, C.H. Kuo, E. Torres, C.M. Niemeyer, Apoenzyme reconstitution as a chemical tool for structural enzymology and biotechnology, Angew. Chemie (International Ed.in English). 48 (2009) 1550–1574.

[2] D.P. Heuts, N.S. Scrutton, W.S. McIntire, M.W. Fraaije, What's in a covalent bond? On the role and formation of covalently bound flavin cofactors, FEBS J. 276 (2009) 3405–3427.

[3] P. Macheroux, B. Kappes, S.E. Ealick, Flavogenomics - a genomic and structural view of flavin-dependent proteins, FEBS J. 278 (2011) 2625–2634.

[4] M.H. Hefti, J. Vervoort, W.J.H. Van Berkel, Deflavination and reconstitution of flavoproteins, Eur. J. Biochem. 270 (2003) 4227–4242.

[5] H.L. Levine, Y. Nakagawa, E.T. Kaiser, Flavopapain: Synthesis and properties of semi-synthetic enzymes, Biochem. Biophys. Res. Commun. 76 (1977) 64–70.

[6] G. de Gonzalo, C. Smit, J. Jin, A.J. Minnaard, M.W. Fraaije, Turning a riboflavin-binding protein into a self-sufficient monooxygenase by cofactor redesign, Chem. Commun. (Camb). 47 (2011) 11050–2.

[7] V. Massey, The chemical and biological versatility of riboflavin, Biochem. Soc. Trans. 28 (2000) 283–296.

[8] D.E. Edmondson, P. Newton-Vinson, The Covalent FAD of Monoamine Oxidase: Structural and Functional Role and Mechanism of the Flavinylation Reaction, Antioxid. Redox Signal. 3 (2001) 789–806.

[9] S. Ghisla, V. Massey, New flavins for old: artificial flavins as active site probes of flavoproteins., Biochem. J. 239 (1986) 1–12.

[10] M. Wang, D.L. Roberts, R. Paschke, T.M. Shea, B.S. Masters, J.J. Kim, Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes., Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 8411–8416.

[11] A. Mees, T. Klar, P. Gnau, U. Hennecke, A.P.M. Eker, T. Carell, et al., Crystal structure of a photolyase bound to a CPD-like DNA lesion after in situ repair., Science. 306 (2004) 1789–1793.

[12] J.R. Miller, D.E. Edmondson, Influence of flavin analogue structure on the catalytic activities and flavinylation reactions of recombinant human liver monoamine oxidases A and B, J. Biol. Chem. 274 (1999) 23515–23525.

[13] C.T. Moonen, F. Müller, On the mobility of riboflavin 5'-phosphate in *Megasphaera elsdenii* flavodoxin as studied by 13C-nuclear-magnetic-resonance relaxation., Eur. J. Biochem. 133 (1983) 463–470.

[14] C.T. Moonen, J. Vervoort, F. Müller, Reinvestigation of the structure of oxidized and reduced flavin: carbon-13 and nitrogen-15 nuclear magnetic resonance study., Biochemistry. 23 (1984) 4859–4867.

[15] J. Vervoort, F. Müller, S.G. Mayhew, W.A. van den Berg, C.T. Moonen, A. Bacher, A comparative carbon-13, nitrogen-15, and phosphorus-31 nuclear magnetic resonance study on the flavodoxins from *Clostridium MP*, *Megasphaera elsdenii*, and *Azotobacter vinelandii*., Biochemistry. 25 (1986) 6789–6799.

[16] G. Fleischmann, F. Lederer, F. Müller, A. Bacher, H. Rüterjans, Flavin-protein interactions in flavocytochrome b2 as studied by NMR after reconstitution of the enzyme with 13C- and 15N-labelled flavin., Eur. J. Biochem. 267 (2000) 5156–5167.

[17] M. Salomon, W. Eisenreich, H. Dürr, E. Schleicher, E. Knieb, V. Massey, et al., An optomechanical transducer in the blue light receptor phototropin from *Avena sativa*., Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 12357–12361.

[18] C. Sanner, P. Macheroux, H. Rüterjans, F. Müller, A. Bacher, 15N- and 13C-NMR investigations of glucose oxidase from *Aspergillus niger*., Eur. J. Biochem. 196 (1991) 663–672.

[19] B.J. Stockman, W.M. Westler, E.S. Mooberry, J.L. Markley, Flavodoxin from *Anabaena 7120*: uniform nitrogen-15 enrichment and hydrogen-1, nitrogen-15, and phosphorus-31 NMR investigations of the flavin mononucleotide binding site in the reduced and oxidized states., Biochemistry. 27 (1988) 136–142.

[20] J. Vervoort, W.J. Van Berkel, F. Müller, C.T. Moonen, NMR studies on p-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* and salicylate hydroxylase from *Pseudomonas putida*., Eur. J. Biochem. 200 (1991) 731–738.

[21] W.D. Beinert, H. Rüterjans, F. Müller, Nuclear magnetic resonance studies of the old yellow enzyme. 1. ^{15}N NMR of the enzyme recombined with ^{15}N-labeled flavin mononucleotides., Eur. J. Biochem. 152 (1985) 573–579.

[22] H.-D. Franken, H. Rüterjans, F. Müller, Nuclear-magnetic-resonance investigation of ^{15}N-labeled flavins, free and bound to *Megasphaera elsdenii* apoflavodoxin, Eur J Biochem. 138 (1984) 481–489.

[23] R. Miura, Y. Miyake, 13C-NMR studies on the reaction intermediates of porcine kidney D-amino acid oxidase reconstituted with ^{13}C-enriched flavin adenine dinucleotide., J. Biochem. 102 (1987) 1345–1354.

[24] J. Kim, J.H. Fuller, V. Kuusk, L. Cunane, Z.W. Chen, F.S. Mathews, et al., The cytochrome subunit is necessary for covalent FAD attachment to the flavoprotein subunit of *p*-cresol methylhydroxylase, J. Biol. Chem. 270 (1995) 31202–31209.

[25] J.W. Koetter, G.E. Schulz, Crystal structure of 6-hydroxy-D-nicotine oxidase from *Arthrobacter nicotinovorans*, J. Mol. Biol. 352 (2005) 418–428.

[26] J. Jin, H. Mazon, R.H. van den Heuvel, A.J. Heck, D.B. Janssen, M.W. Fraaije, Covalent flavinylation of vanillyl-alcohol oxidase is an autocatalytic process, FEBS J. 275 (2008) 5191–5200.

[27] P. Trickey, M.A. Wagner, M.S. Jorns, F.S. Mathews, Monomeric sarcosine oxidase: structure of a covalently flavinylated amine oxidizing enzyme, Structure. 7 (1999) 331–345.

[28] I. Efimov, W.S. McIntire, A study of the spectral and redox properties and covalent flavinylation of the flavoprotein component of *p*-cresol methylhydroxylase reconstituted with FAD analogues, Biochemistry. 43 (2004) 10532–10546.

[29] F.J. Bullock, O. Jardetzky, An Experimental Demonstration of the Nuclear Magnetic Resonance Assignments in the 6,7-Dimethylisoalloxazine Nucleus, J. Org. Chem. 30 (1965) 2056–2057.

[30] C. Walsh, Flavin Coenzymes: At the Crossroads of Biological Redox Chemistry, Acc. Chem. Res. 13 (1980) 148–155.

[31] S. V Bandrin, P.M. Rabinovich, A.I. Stepanov, 3 linkage groups of the genes of riboflavin biosynthesis in *Escherichia coli*, Genetika. 19 (1983) 1419–1425.

[32] A.S. Eisenberg, J.P.M. Schelvis, Contributions of the 8-methyl group to the vibrational normal modes of flavin mononucleotide and its 5-methyl semiquinone radical., J. Phys. Chem. A. 112 (2008) 6179–89.

CHAPTER 6

FUNCTIONAL ROLE OF THE ACTIVE SITE GLU324 OF PUTRESCINE OXIDASE

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ABSTRACT

Putrescine oxidase (PuO) catalyses the oxidative deamination of putrescine into 4-aminobutanal. An increase in diamine carbon chain length decreases the catalytic efficiency (k_{cat}/K_M) significantly. The recently elucidated PuO structure has shown that Glu324 seems to be important for determining the distance between the flavin and the binding site for the positively charged substrate amino group. A PuO mutant, E324D, was produced and showed similar activity for putrescine and cadaverine.

INTRODUCTION

Flavoprotein oxidases belong to a family of oxidases that bind FAD or FMN as a prosthetic group. They catalyse the two- or four-electron oxidation on a wide variety of substrates, such as carbohydrates, alcohols, and amines. Oxidases use molecular oxygen as the electron acceptor, which is reduced to hydrogen peroxide or water during the reaction. Consequently, they do not need expensive coenzymes (e.g. NAD(P)H). What is more, they usually selectively oxidize their substrate at a specific position, while other positions stay unchanged. These two properties make them ideal for biotechnological applications. For example, a fungal monoamine oxidase from *Aspergillus niger* is used as biocatalyst for the deracemization of secondary and tertiary amines [1,2]. Another amine oxidase, D-amino acid oxidase, has been applied in several biotechnological processes, like production of semisynthetic antibiotics and α-keto acids [3].

Amine oxidations are also important in many biological processes, such as lysyl oxidation in the cross-linking of collagen and degradation of polyamines and neurotransmitters. Flavin amine oxidases perform oxidative cleavage of the α-CH bond of the substrate, coupled with the reduction of FAD. An imine, the product of the reaction, is then hydrolysed to the corresponding ketone/aldehyde and ammonia. The reduced flavin cofactor reoxidizes spontaneously in the presence of molecular oxygen and hydrogen peroxide is formed. Most flavin-containing amine oxidases share several structural features. The active site is typically situated on the *re* side of the flavin ring. The substrate amine group is located between two aromatic rings forming an "aromatic sandwich" [4], with typical combination of Phe/Tyr or Tyr/Tyr, as found for monoamine oxidases and polyamine oxidase [5-7]. Another structural motif shared among amine and amino acid oxidases is the "Lys-H_2O-flavin N5" element, where a water molecule acts as a bridge between lysine and the flavin N5 atom [4].

Putrescine oxidase from *Rhodococcus erythropolis* (PuO) is a recently discovered amine oxidase, which catalyses the oxidative deamination of putrescine into 4-aminobutanal [8]. In addition to putrescine, activity was found with several aliphatic diamines (cadaverine, 1,6-hexane-diamine), amino alcohols (4-amino-1-butanol, L-ornithine, 2-hydroxyputrescine) and polyamines (spermidine, spermine). However, an increase in diamine carbon chain length from 4 to 5 C-atoms decreases the catalytic efficiency (k_{cat}/K_M) 15 times and from 4 to 6 C-atoms >1000 times.

Shorter diamines (ethylenediamine, 1,3-diaminopropane) and monoamines (*n*-butylamine, aminoethanol) were shown to be strong inhibitors of PuO. It has been suggested that a minimum of two amino groups, preferably in a distance of 4 carbons from each other, are necessary to be a good substrate for the enzyme [8]. The narrow substrate specificity and high selectivity for putrescine can be well explained by the recently elucidated structure of PuO active site (Fig. 6.1) [9].

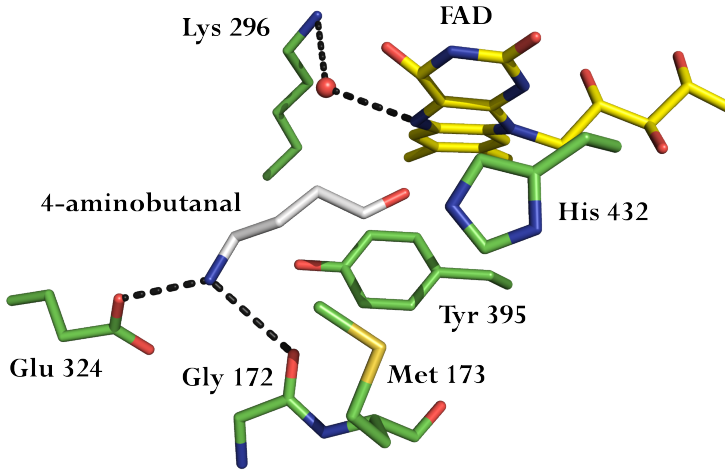

Figure 6.1. The crystal structure of the PuO active site with a bound ligand, modelled as 4-aminobutanal. Oxygens are in red and nitrogens in blue. The protein carbon atoms are in green, FAD carbons are in yellow, whereas ligand carbon atoms are in grey.

PuO exhibits all above-mentioned structural features of amine oxidases. It has a bipartite cavity with approximately equal inner and outer chambers. Putrescine occupies the inner space, where its amino group forms H-bonds with the side chain of Glu324 and the carbonyl oxygen of Gly172. Together with Met173, these two residues make the constriction that separates the two chambers. Glu324 seems to be important for determining the distance between the flavin and the binding site for the positively charged substrate amino group. That explains why PuO prefers diamino substrates with amino groups separated by four and five C-atoms. It also suggests that Glu324 PuO mutants can be generated with altered substrate acceptance profiles, e.g. PuO acting on longer aliphatic diamines and polyamines. The importance of Glu324 was previously investigated to some extent [8]. The amino acid was mutated into an alanine (PuO E324A) and a leucine residue

(PuO E324L). For both mutants the catalytic efficiency for putrescine decreased dramatically: 100,000 and 200,000-fold, respectively. In this study, we report on a PuO mutant, E324D, that shows similar activity for putrescine and cadaverine.

MATERIALS AND METHODS

Oligonucleotides and horseradish peroxidase were from Sigma-Aldrich. *PfuTurbo* DNA polymerase was from Stratagene. *Escherichia coli* TOP10 from Invitrogen was used as a host for DNA manipulation and protein expression. All other chemicals were of analytical grade. The construct was sequenced at GATC Biotech (Konstanz, Germany).

The mutant was prepared using the QuikChange site-directed mutagenesis kit from Stratagene. pBAD-PuO_{Rh} [8] was the template. The primers used for this were E324D_frw, 5'- GAGGTAGTGCAGGACGTGTACGACAACAC-3'; E324D_rv, 5'- GTGTTGTCGTACACGTCCTGCACTACCTC -3'. The sites of mutation are underlined.

E. coli TOP10 cells containing pBAD-PuO_{Rh}-E324D were tested for overexpression of the protein at 17, 30 and 37°C. The range of L-(+)-arabinose concentration was 0, 0.0002, 0.002, 0.02, 0.2 and 2% (wt/vol). Soluble and insoluble fractions of cell extracts were analysed by SDS-PAGE to determine the best conditions of soluble PuO E324D expression. *E. coli* TOP10 containing pBAD-PuO_{Rh}-E324D were cultivated for 3 days at 17°C in 1 L terrific broth medium containing 50 µg/mL ampicillin and 0.002% (wt/vol) L-(+)-arabinose. Protein purification was performed as described before using 50 mM KPi pH 8.0 as buffer [8].

Absorbance spectra were recorded in 50 mM KPi buffer pH 8.0 on a PerkinElmer Lambda Bio40 spectrophotometer. Enzyme concentration was determined by measurement of absorbance at 280 nm using ε_{280} = 63,370 M^{-1} cm^{-1} (www.expasy.ch/sprot/) and recalculated for monomers containing FAD. FAD/ADP content in PuO E324D was assumed to be as for wild-type PuO [10]. Oxidase activity was measured by the use of the coupled horseradish peroxidase assay as described before at pH 8.0 [8] on a SynergyMx (Biotek) plate reader. The activity data were fitted with SigmaPlot 11.0.1 using the Michaelis-Menten equation.

RESULTS & DISCUSSION

In order to change PuO specificity from putrescine toward cadaverine, the mutant PuO E324D was constructed, expressed and purified. The protein purity was confirmed by SDS-PAGE analysis. The mutant shows a typical flavoprotein spectrum, that resembles the one of wild-type PuO [8]. The steady-state kinetic parameters with putrescine and cadaverine were determined at pH 8.0 for wild-type and mutant PuO (Table 6.1). It is clear that wild-type PuO highly prefers putrescine over cadaverine as a substrate. An increase in carbon length of the diamine from 4 to 5 C-atoms resulted in a 25-fold drop in catalytic efficiency (k_{cat}/K_M). The values are highly similar for the ones obtained previously in Tris buffer [8]. It was suggested that the preferred carbon chain length between two amine groups is determined by Glu324: the Glu324-flavin distance determines PuO specificity toward diamines and polyamines with two amine groups separated by four carbons. Therefore, a decrease in carbon length of the active site residue by one C-atom was expected to result in a change of PuO specificity toward the substrates with five carbons between the two amine groups (cadaverine). Our results with E324D PuO confirm this assumption. The mutant does not show preference for putrescine as it is equally active with both cadaverine and putrescine. What is more, the K_M for cadaverine is lower, which suggests that the mutant has a higher affinity toward the five-carbon substrate. However, the turnover rate (k_{cat}) of E324D PuO is lower with cadaverine than with putrescine, and also lower when compared to wild-type PuO with these substrates. This may suggest that Glu324 is somehow better positioned in the active centre to accommodate productive binding of diamine substrates.

Table 6.1. Steady-state kinetic parameters of wild-type and E324D PuO, measured in KPi buffer, pH 8.0.

	wild-type			E324D		
substrate	k_{cat} [s^{-1}]	K_M [μM]	k_{cat}/K_M [mM^{-1} s^{-1}]	k_{cat} [s^{-1}]	K_M [μM]	k_{cat}/K_M [mM^{-1} s^{-1}]
putrescine	21.0±0.06	6.4±0.8	3300±350	4.2±0.1	55.7±3.6	75±4
cadaverine	2.8±0.03	20.9±0.9	130±4	1.6±0.01	23.8±0.4	66±1

Before the structure of PuO was known, the presence of a negatively charged amino acid in the active site was already predicted for PuO from *Micrococcus rubens*, as the enzyme was irreversibly inactivated by carbodiimides [11]. The importance of Glu324 in PuO from *Rhodococcus erythropolis* was confirmed by its mutation to alanine and leucine [8]. The respective single PuO mutants, E324A and E324L, were shown to be poorly active with putrescine. Their catalytic efficiency at pH 9.0 drops from 5900 mM^{-1} s^{-1} for wild-type PuO to 0.05 mM^{-1} s^{-1} and 0.03 mM^{-1} s^{-1} for E324A and E324L PuO, respectively. The most significant is the increase in K_M value for more than 30,000-fold for both mutants, when compared to wild-type enzyme. Together with our results on the E324D PuO mutant, it fully confirms the significance of a carefully positioned negative charge in active site. When the charge is retained but positioned more distant from the flavin cofactor, the enzyme still shows significant activity with putrescine and cadaverine. Nevertheless it loses the ability to discriminate between these two diamines. When the negative charge is removed (e.g. E324A PuO), a dramatic loss of activity is observed. A BLAST search in the available sequence databases of NCBI reveals 17 putrescine oxidases or probable putrescine oxidases and all of them have glutamate at the same position as PuO from *Rhodococcus erythropolis*. Other more distant homologs seem to have rather a hydrophobic corresponding residue. It suggests that the glutamate is a conserved residue that can be used to annotate putrescine oxidase sequences.

REFERENCES

[1] R. Carr, M. Alexeeva, M.J. Dawson, V. Gotor-Fernandez, C.E. Humphrey, N.J. Turner, Directed evolution of an amine oxidase for the preparative deracemisation of cyclic secondary amines, ChemBioChem. 6 (2005) 637-639.

[2] C.J. Dunsmore, R. Carr, T. Fleming, N.J. Turner, A chemo-enzymatic route to enantiomerically pure cyclic tertiary amines, J. Am. Chem. Soc. 128 (2006) 2224-2225.

[3] M.S. Pilone, L. Pollegioni, D-amino acid oxidase as an industrial biocatalyst, Biocatal. Biotransform. 20 (2002) 145-159.

[4] C. Binda, A. Mattevi, D.E. Edmondson, Structure-function relationships in flavoenzyme-dependent amine oxidations: a comparison of polyamine oxidase and monoamine oxidase, J. Biol. Chem. 277 (2002) 23973-23976.

[5] D.E. Edmondson, C. Binda, J. Wang, A.K. Upadhyay, A. Mattevi, Molecular and mechanistic properties of the membrane-bound mitochondrial monoamine oxidases, Biochemistry. 48 (2009) 4220-4230.

[6] K.E. Atkin, R. Reiss, V. Koehler, et al., The structure of monoamine oxidase from *Aspergillus niger* provides a molecular context for improvements in activity obtained by directed evolution, J. Mol. Biol. 384 (2008) 1218-1231.

[7] C. Binda, R. Angelini, R. Federico, P. Ascenzi, A. Mattevi, Structural bases for inhibitor binding and catalysis in polyamine oxidase, Biochemistry. 40 (2001) 2766-2776.

[8] E.W. van Hellemond, M. van Dijk, D.P. Heuts, D.B. Janssen, M.W. Fraaije, Discovery and characterization of a putrescine oxidase from *Rhodococcus erythropolis* NCIMB 11540, Appl. Microbiol. Biotechnol. 78 (2008) 455-463.

[9] M.M. Kopacz, S. Rovida, E. van Duijn, M.W. Fraaije, A. Mattevi, Structure-based redesign of cofactor binding in putrescine oxidase, Biochemistry. 50 (2011) 4209-4217.

[10] E.W. van Hellemond, H. Mazon, A.J. Heck, et al., ADP competes with FAD binding in putrescine oxidase, J. Biol. Chem. 283 (2008) 28259-28264.

[11] W.F. Swain, R.J. Desa, Mechanism of action of putrescine oxidase. Binding characteristics of the active site of putrescine oxidase from *Micrococcus rubens*, Biochim. Biophys. Acta. 429 (1976) 331-341.

CHAPTER 7

KINETIC MECHANISM OF PUTRESCINE OXIDASE FROM RHODOCOCCUS ERYTHROPOLIS

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ABSTRACT

Putrescine oxidase from *Rhodococcus erythropolis* (PuO) is a flavin-containing amine oxidase from the monoamine oxidase family that performs oxidative deamination of aliphatic diamines. In this study we report pre-steady-state kinetic analyses of the enzyme with the use of single- and double-mixing stopped-flow spectroscopy and putrescine as a substrate. During the fast and irreversible reductive-half reaction no radical intermediates were observed, suggesting a direct hydride transfer from the substrate to the FAD. The rate of flavin reoxidation depends on the ligand binding; when the product was bound to the enzyme the rate was higher than with free enzyme species. Similar results were obtained with product-mimicking ligands and this indicates that a ternary complex is formed during catalysis. The obtained kinetic data were used together with steady-state rate equations derived for ping-pong, ternary complex and bifurcated mechanisms to explore which mechanism is operative. The integrated analysis revealed that PuO employs a bifurcated mechanism due to comparable rates of product release from the reduced enzyme and reoxidation of the reduced enzyme-product complex.

INTRODUCTION

Redox enzymes catalyse the electron transfer from one substrate to another and generally make use of an enzyme-bound cofactor. Most of the oxidoreductases employ organic cofactors such as flavins. These enzymes are referred to as flavin-containing oxidoreductases and include oxidases, dehydrogenases, reductases and monooxygenases. Flavoprotein oxidases catalyse two-electron oxidations of a wide variety of substrates, including alcohols and amines. They use molecular oxygen as an electron acceptor, which is typically reduced to hydrogen peroxide. This feature makes flavin-containing oxidases interesting biocatalysts, because they do not require an expensive coenzyme (e.g. NAD(P)H). It is also worth noting that the reactions catalysed by flavin-containing enzymes do not always share mechanistic pathways, which distinguish them from enzymes using other cofactors [1].

Amine oxidases are enzymes that oxidize carbon-nitrogen bonds, mostly in amino acids as well as primary and secondary amines. Single C-N bond oxidation leads to the formation of a double bond, which is usually followed by hydrolysis and the loss of ammonium from primary amines or the cleavage of secondary amines. Amine oxidases can be classified based on the chemical identity used for catalysis, namely copper/quinone or a flavin cofactor. Most of the flavin-containing amine oxidases can be divided into two structural families: the monoamine oxidase (MAO) and D-amino acid oxidase (DAAO) families. The MAO family consists of MAO A and B [2,3], L-amino acid oxidases (LAAO) [4,5], spermine and polyamine oxidases [6,7], histone lysine demethylase LSD1 [8,9] and putrescine oxidase (PuO) [10,11]. The DAAO family contains DAAO [12] and enzymes oxidizing glycine or *N*-methylated-amino acids [13-17]. Amine oxidations play many important roles in nature, for example in neurotransmission [18], cell growth and differentiation [19] and neoplastic cell proliferation [20,21], as well as in biocatalytic applications [22,23].

Although the mechanistic features of the copper/quinone-containing oxidases are reasonably well understood [24-26], the debate on flavin-containing amine oxidations still continues. From the many mechanisms proposed over the years, three have gained the greatest support: (i) the concerted polar nucleophilic mechanism [27-29], (ii) the direct hydride transfer mechanism [30,31] and (iii) the single electron transfer mechanism [30,32]. This broad spectrum of possible mechanisms originates from the ability of the flavin to accept one or two electrons and to form adducts with nucleophiles. Regardless of the way in which a hydride

equivalent is transferred from the substrate to the flavin, they all lead to the oxidized substrate and the reduced flavin, and therefore are called reductive-half reactions. In order to close the catalytic cycle the flavin has to transfer electrons to an electron acceptor. This happens in an oxidative-half reaction and oxidases use molecular oxygen as the electron acceptor. Reoxidation of the flavin can take place both before or after the product is released, following a ternary complex or ping-pong mechanism, respectively (Fig. 7.1). Changes in the redox state of the enzyme can be followed by monitoring the flavin absorbance spectra, because oxidized FAD strongly absorbs light at wavelengths around 360 and 450 nm, in contrast to the reduced form of the flavin which shows no absorption at these wavelengths.

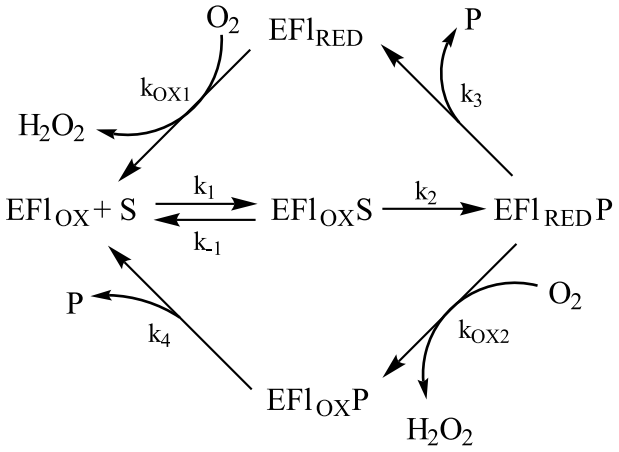


Figure 7.1. Common kinetic mechanisms of flavoprotein oxidases. The upper cycle represents a ping-pong mechanism and the lower cycle a ternary complex mechanism. EFl_{OX} = oxidized form of enzyme, EFl_{RED} = reduced form of enzyme, S = substrate, P = product.

Putrescine oxidase from *Rhodococcus erythropolis* (PuO) belongs to the MAO family of flavin-containing amine oxidases [10] and it catalyses the oxidation of putrescine as well as several other aliphatic diamines, amino alcohols and polyamines. Putrescine is by far the best substrate with a k_{cat} of 26.4 s^{-1} and a K_M of 8.2 μM. The narrow substrate specificity and strong preference for putrescine can be explained by the recently elucidated crystal structure of PuO [11]. The enzyme has a closed bipartite cavity for substrate binding, with Glu324 determining the distance between the positively charged amine group of the substrate and the second amine group close to the flavin. PuO also shares all the characteristic features of

MAO-type oxidases such as an aromatic sandwich, a conserved pattern of interactions with the cofactor and a distorted nonplanar flavin [11,33-35]. Because biogenic amines are derived from free amino acids, PuO and other amine oxidases can be used for their detection in spoiled food [36,37] and in blood as a cancer marker [38].

So far, the kinetic mechanism of PuO has not been established. Here, we present a detailed kinetic analysis of PuO using both single- and double-mixing stopped-flow spectrophotometry. The results provide insight into the amine oxidation performed by PuO and the importance of product and/or ligand binding for the rate of the reoxidation of the protein-bound FAD.

MATERIALS AND METHODS

Chemicals

Escherichia coli TOP10 from Invitrogen was used as a host for DNA manipulation and protein expression. All other chemicals were of analytical grade.

Expression and purification

Expression and purification of PuO was performed according to literature procedures [10].

Stopped-flow experiments

Pre-steady-state experiments were performed on an Applied Photophysics stopped-flow apparatus model SX20. Spectral data were collected at time intervals of 1.2 ms using a photo-diode array (PDA) detector and deconvoluted with the Pro-K software of Applied Photophysics. From this data, the wavelength of 450 nm was selected to follow traces in time with the use of the photomultiplier (PM) detector. All solutions were made anaerobic by flushing with nitrogen for at least 10 min. The anaerobic preparations of PuO were used for transient kinetic analyses and putrescine was used as a substrate. All experiments were performed in a 50 mM KPi buffer pH 8.0 at 25 °C. All data presented in this work is an average value from a minimum of 3-12 measurements.

In all cases the terms reductive and oxidative half-reaction relate to the flavin redox state. In the reductive half-reaction measurements, the concentrations of PuO in the quartz cell were approximately 5 μM and were at least a 5-fold lower than the varying concentrations of putrescine. Equal volumes of anaerobic PuO and putrescine were rapidly mixed in the stopped-flow device and spectral changes were monitored for 1 s using the PDA detector for global analysis and the PM detector at 450 nm to calculate the observed rates. In order to study the oxidative half-reaction, PuO was anaerobically reduced with 1.2-1.6 equivalents of putrescine and incubated for different delay times in sequential mixing stopped-flow aging chamber. Subsequently, the reduced PuO was mixed with air saturated buffer and spectral changes were monitored using a PDA detector. The reoxidation of PuO in the presence of 1 mM ligands, as well as in the absence of ligands, was also measured in a single mixing stopped-flow. Traces obtained by PM measurements were fitted to a single exponential (Eq. 7.1) or double exponential (Eq. 7.2) function, where A(t) represents the absorbance change in time, t is the time in seconds, k_{obs}, $k_{obs,1}$ and $k_{obs,2}$ are the observed rate constants for the singular or the first and second kinetic event, respectively, A and B are corresponding amplitudes and C is the final observed value for the absorbance. The observed rates for substrate concentration dependent reduction of PuO were fitted using Equation 7.3. The observed rates for aging time dependent PuO reoxidation were fitted using Equation 7.4, where t is delay time in aging chamber and a, b and $k_{obs,1}$ are constants.

$$A(t) = A * e^{(-k_{obs}t)} + C \qquad \text{(Eq. 7.1)}$$

$$A(t) = A * e^{(-k_{obs,1}t)} + B * e^{(-k_{obs,2}t)} + C \qquad \text{(Eq. 7.2)}$$

$$k_{obs} = \frac{k_{red}*S}{K_d+S} \qquad \text{(Eq. 7.3)}$$

$$k_{obs} = a * e^{-b*t} + k_{obs,1} \qquad \text{(Eq. 7.4)}$$

Enzyme monitored turnover (EMT) experiments were performed by aerobically mixing equal volumes of approximately 10 μM PuO in KPi buffer and 2 mM putrescine in KPi buffer. The change in absorbance was followed at 450 nm for 60 seconds.

Mechanistic calculations

From the kinetic scheme proposed in Figure 7.1, equations for the change in concentration of each enzyme species were derived for the ping-pong, ternary complex and bifurcated mechanisms. Under steady-state conditions the concentration of each species remains constant and therefore all equations equal zero. The equations were solved using the MuPAD Symbolic Math Toolbox add-on for MATLAB 5.9.0 software for non-trivial solution. The obtained concentrations of the enzyme species were used in the steady-state rate equation in the form of a Michaelis-Menten equation (Eq. 7.5), ping-pong (Eq. 7.6), ternary complex (Eq. 7.7) and bifurcated mechanism (Eq. 7.8), where E_R, E_{OX}, $E_{OX}S$, E_RP and $E_{OX}P$ correspond to enzyme-flavin redox state in Figure 7.1.

$$\frac{v}{[E_T]} = \frac{k_{cat}*[S]}{K_M+[S]} \quad \text{(Eq. 7.5)}$$

$$\frac{v}{[E_T]} = \frac{k_3*[E_RP]}{[E_{ox}]+[E_{ox}S]+[E_RP]+[E_R]} \quad \text{(Eq. 7.6)}$$

$$\frac{v}{[E_T]} = \frac{k_4*[E_{ox}P]}{[E_{ox}]+[E_{ox}S]+[E_RP]+[E_{ox}P]} \quad \text{(Eq. 7.7)}$$

$$\frac{v}{[E_T]} = \frac{k_3*[E_RP]+k_4*[[E_{ox}P]}{[E_{ox}]+[E_{ox}S]+[E_RP]+[E_{ox}P]+[E_R]} \quad \text{(Eq. 7.8)}$$

From these rate equations, the kinetic parameters K_M and k_{cat} can be derived and the redox state of the flavin in steady-state conditions can be calculated:

Ping-pong mechanism:

$$k_{cat} = \frac{k_2*k_3*k_{ox1}*[O_2]}{k_2*k_3+(k_2+k_3)k_{ox1}*[O_2]} \quad \text{(Eq. 7.9)}$$

$$K_M = \frac{k_3*k_{ox1}*[O_2]*K_D}{k_2*k_3+(k_2+k_3)k_{ox1}*[O_2]} \quad \text{(Eq. 7.10)}$$

$$\%OX = \frac{([S]+K_D)k_3*k_{ox1}*[O_2]}{k_2*k_3*[S]+(k_2+k_3)k_{ox1}*[O_2]*[S]+k_3*k_{ox1}*[O_2]*K_D} * 100 \quad \text{(Eq. 7.11)}$$

Ternary complex mechanism:

$$k_{cat} = \frac{k_2 * k_4 * k_{ox2} * [O_2]}{k_2 * k_4 + (k_2 + k_4) k_{ox2} * [O_2]} \quad \text{(Eq. 7.12)}$$

$$K_M = \frac{k_4 * k_{ox2} * [O_2] * K_D}{k_2 * k_4 + (k_2 + k_4) k_{ox2} * [O_2]} \quad \text{(Eq. 7.13)}$$

$$\%OX = \frac{(k_2 * [S] + [O_2] * [S] + k_4 * K_D) k_{ox2} * [O_2]}{k_2 * k_4 * [S] + (k_2 + k_4) k_{ox2} * [O_2] * [S] + k_4 * k_{ox2} * [O_2] * K_D} * 100 \quad \text{(Eq. 7.14)}$$

Bifurcated mechanism:

$$k_{cat} = \frac{k_2 * k_4 * k_{ox1} * [O_2] (k_3 + k_{ox2} * [O_2])}{k_2 * k_3 * k_4 + (k_2 + k_3 + k_{ox2} * [O_2]) k_4 * k_{ox1} * [O_2] + k_2 * k_{ox1} * k_{ox2} * [O_2]^2} \quad \text{(Eq. 7.15)}$$

$$K_M = \frac{k_4 * k_{ox1} * [O_2] (k_3 + k_{ox2} * [O_2]) K_D}{k_2 * k_3 * k_4 + (k_2 + k_3 + k_{ox2} * [O_2]) k_4 * k_{ox1} * [O_2] + k_2 * k_{ox1} * k_{ox2} * [O_2]^2} \quad \text{(Eq. 7.16)}$$

$$\%OX = \frac{1 + \frac{K_D}{[S]} + \frac{k_2 * k_{ox2} [O_2]}{k_4 (k_3 + k_{ox2} * [O_2])}}{1 + \frac{k_2}{k_3 + k_{ox2} * [O_2]} + \frac{K_D}{[S]} + \frac{k_2 * k_3}{k_{ox1} * [O_2] (k_3 + k_{ox2} * [O_2])} + \frac{k_2 * k_{ox2} [O_2]}{k_4 (k_3 + k_{ox2} * [O_2])}} * 100 \quad \text{(Eq. 7.17)}$$

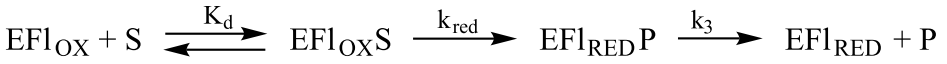
Analytical methods

Absorbance spectra were recorded in a 50 mM KPi pH 8.0 buffer at 25 °C on a PerkinElmer Lambda Bio40 spectrophotometer. Enzyme concentrations were determined by measurement of the absorbance at 280 nm using $\varepsilon_{280} = 63,370\ M^{-1}cm^{-1}$ (http://www.expasy.ch/sprot/) and recalculated for monomers containing FAD [11]. The oxidase activity was measured with the use of a horseradish peroxidase coupled assay as described in literature at pH 8.0 [10].

RESULTS

The reductive half-reaction

In order to determine the kinetics of flavin reduction in PuO (Scheme 7.1) single mixing stopped-flow spectrophotometry was used. Initially the reductive half-reaction was monitored with a photodiode array (PDA) upon mixing PuO with varying concentrations of putrescine in the absence of oxygen. Deconvolution of the collected spectra revealed that the spectrum of oxidized flavin rapidly changed into a spectrum that resembles a fully reduced flavin species and no radical flavin intermediates were observed. Thus, if any flavin radical intermediate was formed during the PuO-catalysed oxidation, its concentration was too low to allow spectral detection. The fast flavin reduction is followed by a slow phase which is associated with a significantly smaller absorbance change. For more accurate determination of the rates of these two kinetic events, the reductive half-reactions were also monitored with a photomultiplier (PM) detector set at 450 nm. The collected traces could be fitted with a double exponential equation (Eq. 7.2). The first phase, the rate of reduction, was dependent on the substrate concentration. This indicates that the binding of putrescine is a relatively fast step, because it is complete within the dead time of the stopped-flow instrument (approx. 1 ms). Fitting the observed reduction rates at different putrescine concentrations (Fig. 7.2) could be performed with Equation 7.3, which indicates that flavin reduction is an irreversible process. From the data the rate of reduction (k_{red}) and K_d could be determined: $k_{red} = 252 \pm 8\ s^{-1}$ and $K_d = 82 \pm 16\ \mu M$. The second phase observed during the reductive half-reaction is much slower ($5\ s^{-1}$) and appears to be independent of the substrate concentration. This has also been observed for other flavoprotein oxidases where a relatively slow kinetic event follows the flavin reduction. This second phase usually involves only marginal spectral changes and it is often suggested to represent product release and/or a conformational change [39,40]. The observed rates of this slow event ($5\ s^{-1}$) are relatively low compared to the k_{cat} ($26.4\ s^{-1}$), indicating that this event cannot be a major contributor to the kinetic mechanism of PuO.



Scheme 7.1. Proposed model for the reductive half-reaction of PuO. EFl_{OX} represents the oxidized form of PuO, S the substrate, EFl_{RED} the reduced form of PuO and P the product.

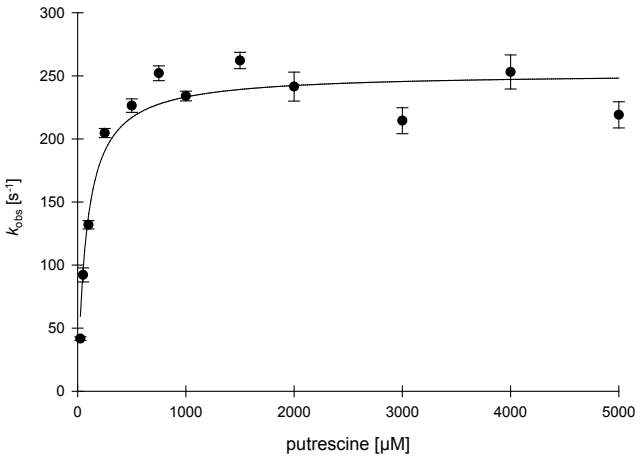
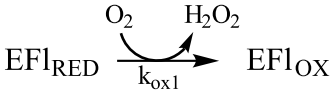


Figure 7.2. Observed rates for the first phase of the reductive half-reaction plotted against the putrescine concentration. The data was fitted with a simple hyperbolic function (Eq. 3). Error bars represent standard errors from at least 8 measurements.

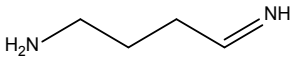
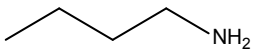
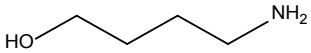
The oxidative half-reaction

Single mixing stopped-flow spectrophotometry was also used to determine the kinetics of the reoxidation of reduced PuO. In order to analyse the reaction of reduced free enzyme (not in complex with the product) with molecular oxygen (Scheme 7.2), PuO was first anaerobically reduced with 1.2 equivalents of putrescine. The sample was incubated for at least 5 min to assure full flavin reduction and release of the product from the enzyme, after which it was mixed with air saturated buffer and spectra were obtained with the PDA detector. From the analysis of these experiments, the enzyme reoxidation turned out to be a single event and no intermediates (e.g. flavin semiquinone) were observed and the reaction was further analysed with the PM detector at 450 nm. The obtained absorbance traces were fitted with Equation 7.1. The observed first-order rate constant for the reaction with oxygen in air saturated buffer when mixed in a 1:1 ratio with anaerobic enzyme/product solution was 6.6 ± 0.5 s^{-1} (Table 7.1).



Scheme 7.2. The proposed model for the oxidative half-reaction of reduced free PuO species. EFl_{RED} represents the reduced form and EFl_{OX} oxidized form of PuO.

Table 7.1. The rate of reoxidation of reduced PuO with or without a ligand bound to the active site. Anaerobic reduced enzyme/ligand solutions were mixed in 1:1 ratio with O_2-containg buffer.

ligand		k_{ox} [s^{-1}]
–	–	6.6 ± 0.5
– [a]	–	5.3 ± 1.4
putrescine-imine [a]		30.9 ± 2.3
n-butylamine [1.0 mM] [b]		15.8 ± 0.3
4-amino-1-butanol [1.0 mM] [b]		15.3 ± 0.3

[a], these rates were measured by using the double-mixing stopped-flow mode, see Fig. 7.4.

[b], a final concentration of 1.0 mM was used for these ligands.

In order to determine the reoxidation rate of the reduced enzyme-product complex ($EFl_{RED}P$), the enzyme is usually pre-incubated with the product before it is reduced. However, in case of PuO this approach was not possible because the unstable imine product rapidly reacts with water, as it has been shown for the opposite reaction of aldehyde and ammonia to give imine and water. The reaction is thermodynamically difficult unless water is removed. For that reason, sequential stopped-flow was used, where PuO was first mixed with 1.6 equivalents of putrescine in an aging chamber in the absence of oxygen to form the reduced complex. The solution was further mixed with an air saturated buffer after different delay times, ranging from 0.05 s to 10 s. From the determined reduction rates it could be estimated that the shortest delay time was still sufficient for full enzyme reduction (>99%) before mixing with oxygen. The spectral changes were monitored with the PDA detector. Again, no clear flavin radical intermediates

could be observed. Yet, depending on the aging time, one or two phases in flavin reoxidation were observed upon deconvolution of the spectra. For the short aging times (0.05 to 4.0 s), two phases could be distinguished, while for the longer aging times (5.0 to 10 s) only one phase was found (Fig. 7.3). The observed rates of oxidation at these long aging times were approximately 5 s^{-1}. At the shorter aging times, the rate of the first phase depended on the delay time in the aging chamber and when relatively short times were used a higher rate was found. When the observed rates were plotted as a function of the delay time, the data could be fitted with a single exponential decay equation (Eq. 7.4, Fig. 7.4). Because the measurements at different delay times represent oxidation of the reduced enzyme at different levels of occupation with the product or decayed product, the reoxidation rates for the reduced enzyme (Scheme 7.2) and the reduced enzyme-product complex (Scheme 7.3) could be obtained from the single exponential decay fit of the observed rate of reoxidation. At a long delay time the product is released from the active site, while the rates at a short delay time resemble reoxidation of the product bound to the reduced enzyme. After fitting the data, the rate of reoxidation of the product bound to the reduced enzyme ($EFl_{RED}P$) could be extrapolated (30.9 s^{-1}) and the rate of reoxidation of the reduced free enzyme (EFl_{RED}) (5.3 s^{-1}) could be derived (Fig. 7.4). The observed rate of the slow second phase varied between 1-3 s^{-1} and was independent of the delay time in the aging chamber. Such a slow observed rate is considered too low to be of significant catalytic relevance. From these rates, the observed bimolecular rates of k_{ox1} = 4.3 x 10^4 M^{-1} s^{-1} and k_{ox2} = 2.5 x 10^5 M^{-1} s^{-1} could be calculated. These values are in the typical range found for oxidases [41] and they suggest that $EFl_{RED}P$ reacts significantly faster with molecular oxygen when compared with EFl_{RED}.

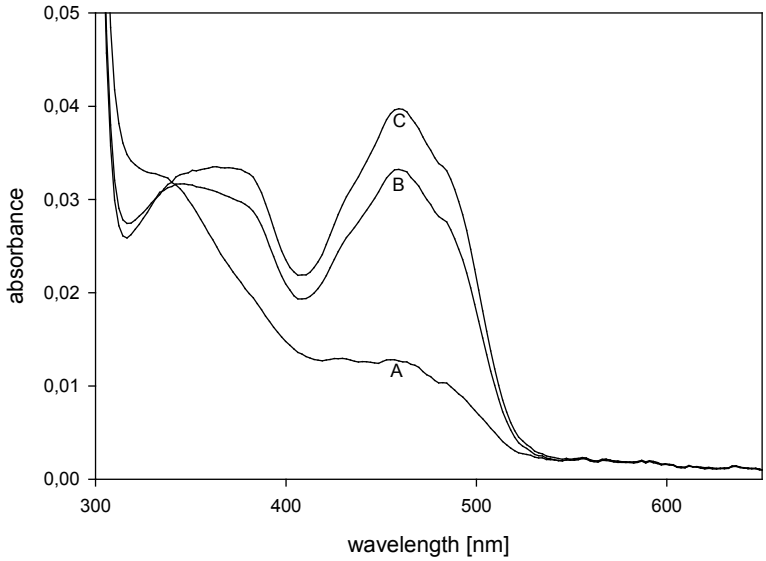


Figure 7.3. Deconvoluted absorption spectra of reduced **(A)**, intermediate **(B)** and oxidized **(C)** PuO during reoxidation by molecular oxygen as measured by double-mixing stopped-flow spectrophotometry, after a delay time of 4.0 s.

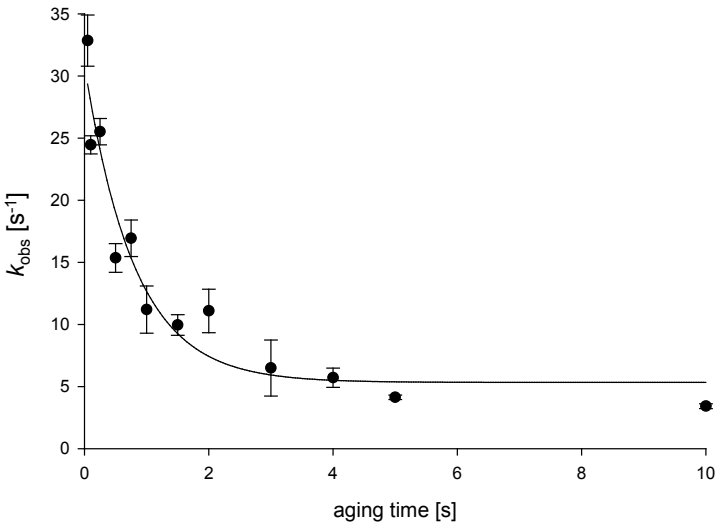
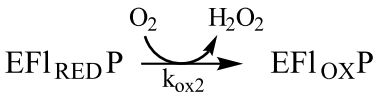


Figure 7.4. Observed rates for the first phase of the oxidative half-reaction plotted against the delay time in the aging chamber in double-mixing stopped-flow mode. Data points represent averages from at least 3 measurements and the error bars are standard errors. The data could be fitted with a single exponential equation (Eq.7.4) yielding a rate at t=0 (30.9 ± 2.3 s^{-1}), a rate at t=∞ (5.3 ± 1.4 s^{-1}), and a decay rate (1.3 ± 0.3 s^{-1}).



Scheme 7.3. The proposed model for the oxidative half-reaction of the reduced PuO species occupied with product. EFl_{RED} represents the reduced form and EFl_{OX} oxidized form of PuO.

To further confirm the observation that PuO is more readily reoxidized when the active site is occupied with a ligand (Scheme 7.3), the substrate-reduced enzyme was incubated for 5 min with an excess of product-mimicking ligand before mixing with dioxygen. This would assure that the enzyme is reduced and complexed with a putrescine-like product analogue before monitoring the rate of reoxidation by molecular oxygen. *n*-Butylamine, a strong competitive PuO inhibitor (K_I = 120 μM), and 4-amino-1-butanol (a poor substrate) [10] were used as PuO ligands. The measured reoxidation rates were significantly higher than the rate of the reduced free enzyme (Table 7.1). This confirms that ligand binding in PuO indeed increases the rate of reoxidation. These results seem to further support the claim that PuO may preferentially follow a ternary complex mechanism.

Enzyme monitored turnover

Stopped-flow spectrophotometry can also be used to monitor the redox state of the flavin cofactor during steady-state turnover. The extent of flavin reduction under steady-state conditions depends on the relative rates of the reductive and oxidative half-reactions. Figure 7.5 shows the time course for the redox state of the flavin in PuO when mixed with an excess of putrescine. Because the reduction rate is very high, a large part of the flavin is already reduced within the dead-time of the measurement. The subsequently observed steady-state phase is relatively short due to the high k_{cat} of PuO. However, the steady-state phase is clearly visible and it was estimated from 7 measurements that 23 % of the flavin was in the oxidized form during this phase. After that, the flavin was slowly reduced to the fully reduced state due to the depletion of molecular oxygen.

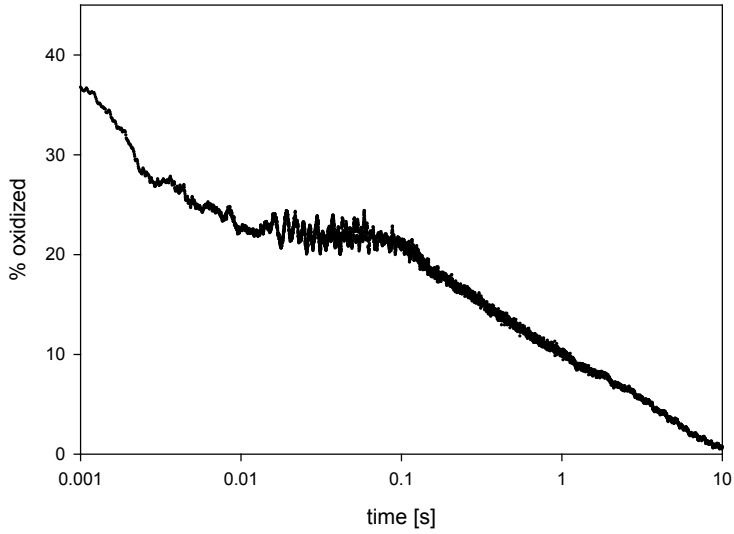


Figure 7.5. Redox state of the flavin in PuO during turnover of putrescine. The reaction was measured at 5.0 μM PuO, 1.0 mM putrescine and 250 μM O_2 and it was monitored at 450 nm. The absorbance of fully oxidized PuO was determined by measuring the absorbance of the enzyme at the same concentration, mixed without substrate. For the sake of clarity, time is presented on a logarithmic scale.

Mechanistic calculations

The kinetic measurements above showed that the rate of PuO reoxidation is significantly higher for the enzyme-product species, suggesting that the enzyme follows a ternary complex mechanism. However, the free enzyme species is also oxidized at an appreciable rate and therefore a ping-pong mechanism may also be (partially) operative during steady-state turnover. In order to address this possibility, we derived the equations for the steady-state kinetic parameters and the redox state of the flavin during the steady-state turnover for both mechanisms, as well as for the bifurcated mechanism (Equations 7.9-7.17). Unfortunately, we could not measure the rates of product release directly, because it was impossible to observe the kinetic event in the pre-steady-state experiments. However, they can be calculated from the experimentally determined kinetic parameters [10] and cofactor redox state (this work). We obtained different values for each of the three mechanisms, suggesting that neither the ping-pong nor the ternary complex mechanism is valid for PuO and a bifurcated mechanism must be operative. The calculated product

release rates for the bifurcated mechanism were k_3 = 13 s^{-1} and k_4 = 181 s^{-1}. With these rates and all determined individual pre-steady-state rates constants, the steady-state kinetic parameters and flavin redox state could be quite accurately predicted using the equations derived for a bifurcated mechanism (Eq. 7.15-7.17 and Table 7.2). When using the product release rates determined for the bifurcated mechanism in the derived equations for the individual mechanisms, significantly less accurate predictions of the respective steady-state parameters were obtained (Table 7.2). Clearly the obtained kinetic data fit best with an enzyme that operates *via* a mixed bifurcated mechanism in which both pathways, namely ping-pong and the ternary complex mechanism, play a significant role. This is due to the fact that the rate of product release from the reduced enzyme is in the same range as the rate of reoxidation of the product-bound enzyme complex. One should note that these conclusions are only valid for conditions at atmospheric oxygen concentrations and with either lower or higher oxygen concentrations, the contribution of each individual type of mechanism will change. In the 1970s the possibility of a mixed mechanism for flavoprotein oxidases was already postulated [42], but remained unsubstantiated until recently when this mechanism was confirmed for human spermine oxidase [39].

Table 7.2. Determined and calculated kinetic parameters for PuO. The calculated values were obtained from the kinetic constants in Fig. 7.1 and the corresponding Equations 7.9-7.17. The values were calculated for k_3 = 13 s^{-1} and k_4 = 181 s^{-1} obtained from the equation for the bifurcated mechanism. The numbers for the redox state represent the percentage of PuO in the oxidized state during the steady-state turnover.

	Experimental	Calculated		
		Ping-pong	Ternary complex	Bifurcated
k_{cat} [s^{-1}]	26.4 ± 0.5[10]	5.7	38.9	26.2
K_M [μM]	8.2 ± 0.5[10]	1.9	12.7	14.7
Redox state [%]	23 ± 1.5	2.5	37.8	23.1

DISCUSSION

The purpose of the current study was to determine the mechanistic properties of putrescine oxidase, a flavin-containing amine oxidase from the MAO family. Members of this flavoprotein oxidase family often display a low level of sequence identity, but their structures are homologous and the flavin binding site is conserved. However, there has been no agreement on the mechanism which amine-oxidizing enzymes follow and a few pathways were proposed for different enzymes (see reviews: [43-48]). Recently, the direct hydride transfer mechanism has gained support, which came mostly from computational studies on lysine-specific demethylase 1 (LSD1) and MAO B [49,50]. Furthermore, the other mechanisms involve the formation of enzyme intermediates with distinct spectral properties and should be detectable in stopped-flow studies. Detection of such intermediates failed for many MAO enzymes [46], including our study on PuO. We have not observed any intermediates in the reductive half-reaction that could hint to alternative mechanisms of amine oxidation. The rate of flavin reduction in PuO is significantly higher than the k_{cat}, indicating that the cleavage of the amine substrate CH bond is not a rate-limiting step, as in the case of MAO A [51,52], MAO B [53] and LSD1 [54].

It is known that flavin reoxidation rates in flavoenzymes often strongly depend on whether the active site of the enzyme is occupied by a ligand. For example, reduced DAAO and MAO are oxidized approximately ten times faster when a product is bound [55,56]. In the case of PuO we observed the same effect. The double-mixing stopped-flow spectrophotometry experiments have clearly shown that the reoxidation of reduced PuO is faster when the product is still bound in the active site, yielding a bimolecular rate constant of 2.5×10^5 M^{-1} s^{-1}. This is one order of magnitude higher than for the reduced free enzyme species. It has been suggested that a proper electrostatic environment in front of the C4a-N5 locus of the flavin can enhance the formation of radical species intermediates [41,57-60]. For DAAO and MAO, the positively charged imine moiety of the bound product is in direct contact with the C4a locus of the FAD [2,11,61]. This might stabilize the canonical form of the reduced flavin with the highest electron density at the C4a locus, which in turn can help in electron transfer to molecular oxygen [61]. The faster reoxidation rate of product-bound PuO compared to reoxidation of the free enzyme species suggests the same function of the bound imine product.

However, increased reoxidation rates were also observed with the other ligands, *n*-butylamine and 4-amino-1-butanol. In their case no positive charge is introduced close to the C4a-N5 locus of the flavin. Yet, the rates were approximately half of the one measured with the product, suggesting that the charge might be not the only factor influencing the reactivity with oxygen. Another determinant for oxygen reactivity may be the oxygen accessibility of the flavin. It has been shown for other flavoproteins that dioxygen molecules diffuse into the active site *via* specific (transient) tunnels [62]. Similarly, ligand binding in PuO may contribute to the accessibility for molecular oxygen to reach the reduced flavin [41,62].

The reoxidation rates measured for PuO suggest that the enzyme preferentially follows a ternary complex mechanism. However, when taking all kinetic parameters into account, it is clear that at atmospheric conditions PuO operates *via* a bifurcated mechanism to which both a ping-pong mechanism and ternary complex mechanism contribute. Such an indecisive mechanism has recently also been found for human spermine oxidase and therefore may be a common feature for amine oxidases. However, one should realize that the contribution of each mechanism strongly depends on the available oxygen concentration. At high oxygen concentrations PuO will mostly follow the ternary complex mechanism, while the ping-pong mechanism becomes more dominant for decreasing O_2 concentrations. Our calculations have shown that the affinity for putrescine is higher in the ping-pong mechanism. There is precedence for this theory, because the actinomycete *R. erythropolis* resides in soil and water environments where the oxygen concentration is relatively low and this might be the reason for the preservation of a less efficient ping-pong mechanism. Whether such an oxygen-dependent switch in the kinetic mechanism is of physiological relevance remains to be established.

REFERENCES

[1] S. Ghisla, V. Massey, Mechanisms of flavoprotein-catalyzed reactions, Eur. J. Biochem. 181 (1989) 1-17.

[2] C. Binda, P. Newton-Vinson, F. Hubálek, D.E. Edmondson, A. Mattevi, Structure of human monoamine oxidase B, a drug target for the treatment of neurological disorders, Nature Structural Biology. 9 (2002) 22-26.

[3] L. De Colibus, M. Li, C. Binda, A. Lustig, D.E. Edmondson, A. Mattevi, Three-dimensional structure of human monoamine oxidase A (MAO A): relation to the structures of rat MAO A and human MAO B, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 12684-12689.

[4] A. Faust, K. Niefind, W. Hummel, D. Schomburg, The Structure of a Bacterial l-Amino Acid Oxidase from *Rhodococcus opacus* Gives New Evidence for the Hydride Mechanism for Dehydrogenation, J. Mol. Biol. 367 (2007) 234-248.

[5] P.D. Pawelek, J. Cheah, R. Coulombe, P. Macheroux, S. Ghisla, A. Vrielink, The structure of L-amino acid oxidase reveals the substrate trajectory into an enantiomerically conserved active site, EMBO J. 19 (2000) 4204-4215.

[6] C. Binda, A. Coda, R. Angelini, R. Federico, P. Ascenzi, A. Mattevi, A 30 Å long U-shaped catalytic tunnel in the crystal structure of polyamine oxidase, Structure. 7 (1999) 265-276.

[7] Q. Huang, Q. Liu, Q. Hao, Crystal structures of Fms1 and its complex with spermine reveal substrate specificity, J. Mol. Biol. 348 (2005) 951-959.

[8] P. Stavropoulos, G. Blobel, A. Hoelz, Crystal structure and mechanism of human lysine-specific demethylase-1, Nature structural & molecular biology. 13 (2006) 626-632.

[9] Y. Chen, Y. Yang, F. Wang, et al., Crystal structure of human histone lysine-specific demethylase 1 (LSD1), Proceedings of the National Academy of Sciences. 103 (2006) 13956-13961.

[10] E.W. van Hellemond, M. van Dijk, D.P. Heuts, D.B. Janssen, M.W. Fraaije, Discovery and characterization of a putrescine oxidase from *Rhodococcus erythropolis* NCIMB 11540, Appl. Microbiol. Biotechnol. 78 (2008) 455-463.

[11] M.M. Kopacz, S. Rovida, E. van Duijn, M.W. Fraaije, A. Mattevi, Structure-based redesign of cofactor binding in putrescine oxidase, Biochemistry. 50 (2011) 4209-4217.

[12] A. Mattevi, M.A. Vanoni, F. Todone, et al., Crystal structure of D-amino acid oxidase: a case of active site mirror-image convergent evolution with flavocytochrome b2, Proceedings of the National Academy of Sciences. 93 (1996) 7496-7501.

[13] E.C. Settembre, P.C. Dorrestein, J. Park, A.M. Augustine, T.P. Begley, S.E. Ealick, Structural and mechanistic studies on ThiO, a glycine oxidase essential for thiamin biosynthesis in *Bacillus subtilis*, Biochemistry (N.Y.). 42 (2003) 2971-2981.

[14] P. Trickey, M.A. Wagner, M.S. Jorns, F.S. Mathews, Monomeric sarcosine oxidase: structure of a covalently flavinylated amine oxidizing enzyme, Structure. 7 (1999) 331-345.

[15] D. Leys, J. Basran, N.S. Scrutton, Channelling and formation of 'active' formaldehyde in dimethylglycine oxidase, EMBO J. 22 (2003) 4038-4048.

[16] A. Ilari, A. Bonamore, S. Franceschini, A. Fiorillo, A. Boffi, G. Colotti, The X-ray structure of N-methyltryptophan oxidase reveals the structural determinants of substrate specificity, Proteins: Structure, Function, and Bioinformatics. 71 (2008) 2065-2075.

[17] P. Khanna, M.S. Jorns, Characterization of the FAD-containing N-methyltryptophan oxidase from *Escherichia coli*, Biochemistry (N.Y.). 40 (2001) 1441-1450.

[18] C.W. Abell, S.W. Kwan, Molecular characterization of monoamine oxidases A and B, Prog. Nucleic Acid Res. Mol. Biol. 65 (2001) 129-156.

[19] T. Thomas, T. Thomas, Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications, Cell. Mol. Life Sci.. 58 (2001) 244-258.

[20] A.E. Pegg, Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy, Cancer Res. 48 (1988) 759-774.

[21] N. Seiler, C. Atanassov, F. Raul, Polyamine metabolism as target for cancer chemoprevention (review). Int. J. Oncol. 13 (1998) 993.

[22] R. Carr, M. Alexeeva, M.J. Dawson, V. Gotor-Fernandez, C.E. Humphrey, N.J. Turner, Directed evolution of an amine oxidase for the preparative deracemisation of cyclic secondary amines, ChemBioChem. 6 (2005) 637-639.

[23] C.J. Dunsmore, R. Carr, T. Fleming, N.J. Turner, A chemo-enzymatic route to enantiomerically pure cyclic tertiary amines, J. Am. Chem. Soc. 128 (2006) 2224-2225.

[24] M. Mure, S.A. Mills, J.P. Klinman, Catalytic mechanism of the topa quinone containing copper amine oxidases, Biochemistry (N.Y.). 41 (2002) 9269-9278.

[25] S. Datta, Y. Mori, K. Takagi, et al., Structure of a quinohemoprotein amine dehydrogenase with an uncommon redox cofactor and highly unusual crosslinking, PNAS. 98 (2001) 14268-14273.

[26] A. Satoh, J. Kim, I. Miyahara, et al., Crystal structure of quinohemoprotein amine dehydrogenase from *Pseudomonas putida* Identification of a novel quinone cofactor encaged by multiple thioether cross-bridges, J. Biol. Chem. 277 (2002) 2830-2834.

[27] M. Li, C. Binda, A. Mattevi, D.E. Edmondson, Functional role of the "aromatic cage" in human monoamine oxidase B: structures and catalytic properties of Tyr435 mutant proteins, Biochemistry. 45 (2006) 4775-4784.

[28] S.S. Erdem, Ö. Karahan, İ. Yıldız, K. Yelekçi, A computational study on the amine-oxidation mechanism of monoamine oxidase: insight into the polar nucleophilic mechanism, Org. Biomol. Chem. 4 (2006) 646-658.

[29] M. Akyüz, S. Erdem, D. Edmondson, The aromatic cage in the active site of monoamine oxidase B: effect on the structural and electronic properties of bound benzylamine and p-nitrobenzylamine, J. Neural Transm. 114 (2007) 693-698.

[30] E.C. Ralph, J.S. Hirschi, M.A. Anderson, W.W. Cleland, D.A. Singleton, P.F. Fitzpatrick, Insights into the mechanism of flavoprotein-catalyzed amine oxidation from nitrogen isotope effects on the reaction of N-methyltryptophan oxidase, Biochemistry (N. Y.). 46 (2007) 7655-7664.

[31] C.W. Kay, H. El Mkami, G. Molla, L. Pollegioni, R.R. Ramsay, Characterization of the covalently bound anionic flavin radical in monoamine oxidase A by electron paramagnetic resonance, J. Am. Chem. Soc. 129 (2007) 16091-16097.

[32] S.E. Rigby, R.M. Hynson, R.R. Ramsay, A.W. Munro, N.S. Scrutton, A stable tyrosyl radical in monoamine oxidase A, J. Biol. Chem. 280 (2005) 4627-4631.

[33] D.E. Edmondson, C. Binda, J. Wang, A.K. Upadhyay, A. Mattevi, Molecular and mechanistic properties of the membrane-bound mitochondrial monoamine oxidases, Biochemistry. 48 (2009) 4220-4230.

[34] K.E. Atkin, R. Reiss, V. Koehler, et al., The structure of monoamine oxidase from *Aspergillus niger* provides a molecular context for improvements in activity obtained by directed evolution, J. Mol. Biol. 384 (2008) 1218-1231.

[35] C. Binda, A. Mattevi, D.E. Edmondson, Structure-function relationships in flavoenzyme-dependent amine oxidations: a comparison of polyamine oxidase and monoamine oxidase, J. Biol. Chem. 277 (2002) 23973-23976.

[36] J. Lee, J. Jang, M. Yu, Y. Kim, Construction of a Bifunctional Enzyme Fusion for the Combined Determination of Biogenic Amines in Foods, J. Agric. Food Chem. 61 (2013) 9118-9124.

[37] B. Bóka, N. Adányi, J. Szamos, D. Virág, A. Kiss, Putrescine biosensor based on putrescine oxidase from *Kocuria rosea*, Enzyme Microb. Technol. (2012).

[38] L. Nagy, G. Nagy, R.E. Gyurcsányi, M.R. Neuman, E. Lindner, Development and study of an amperometric biosensor for the *in vitro* measurement of low concentration of putrescine in blood, J. Biochem. Biophys. Methods. 53 (2002) 165-175.

[39] M.S. Adachi, P.R. Juarez, P.F. Fitzpatrick, Mechanistic studies of human spermine oxidase: kinetic mechanism and pH effects, Biochemistry (N.Y.). 49 (2009) 386-392.

[40] M.S. Adachi, J.M. Torres, P.F. Fitzpatrick, Mechanistic studies of the yeast polyamine oxidase Fms1: kinetic mechanism, substrate specificity, and pH dependence, Biochemistry (N.Y.). 49 (2010) 10440-10448.

[41] A. Mattevi, To be or not to be an oxidase: challenging the oxygen reactivity of flavoenzymes, Trends Biochem. Sci. 31 (2006) 276-283.

[42] D. Porter, H. and Bright, Flavoprotein oxidase mechanisms, in: T. Singer (Ed.), Flavins and Flavoproteins, Elsevier Scientific Pub. Co., 1976, pp. 225-237.

[43] F. Forneris, E. Battaglioli, A. Mattevi, C. Binda, New roles of flavoproteins in molecular cell biology: histone demethylase LSD1 and chromatin, FEBS journal. 276 (2009) 4304-4312.

[44] J.C. Culhane, P.A. Cole, LSD1 and the chemistry of histone demethylation, Curr. Opin. Chem. Biol. 11 (2007) 561-568.

[45] M.W. Fraaije, A. Mattevi, Flavoenzymes: diverse catalysts with recurrent features, Trends Biochem. Sci. 25 (2000) 126-132.

[46] H. Gaweska, P.F. Fitzpatrick, Structures and mechanism of the monoamine oxidase family, Biomolecular concepts. 2 (2011) 365-377.

[47] N.S. Scrutton, Chemical aspects of amine oxidation by flavoprotein enzymes, Nat. Prod. Rep. 21 (2004) 722-730.

[48] P.F. Fitzpatrick, Oxidation of amines by flavoproteins, Arch. Biochem. Biophys. 493 (2010) 13-25.

[49] B. Karasulu, M. Patil, W. Thiel, Amine Oxidation Mediated by Lysine-Specific-Demethylase 1 (LSD1): QM/MM Insights into Mechanism and Role of Lysine 661, J. Am. Chem. Soc. 135, 2013, 13400-13413.

[50] R. Vianello, M. Repič, J. Mavri, How are Biogenic Amines Metabolized by Monoamine Oxidases? European Journal of Organic Chemistry. 2012 (2012) 7057-7065.

[51] J.R. Miller, D.E. Edmondson, Structure-activity relationships in the oxidation of para-substituted benzylamine analogues by recombinant human liver monoamine oxidase A, Biochemistry (N.Y.). 38 (1999) 13670-13683.

[52] R.K. Nandigama, D.E. Edmondson, Structure-activity relations in the oxidation of phenethylamine analogues by recombinant human liver monoamine oxidase A, Biochemistry (N.Y.). 39 (2000) 15258-15265.

[53] M.C. Walker, D.E. Edmondson, Structure-activity relationships in the oxidation of benzylamine analogs by bovine liver mitochondrial monoamine oxidase B, Biochemistry (N. Y.). 33 (1994) 7088-7098.

[54] H. Gaweska, M. Henderson Pozzi, D.M. Schmidt, D.G. McCafferty, P.F. Fitzpatrick, Use of pH and kinetic isotope effects to establish chemistry as rate-limiting in oxidation of a peptide substrate by LSD1, Biochemistry (N.Y.). 48 (2009) 5440-5445.

[55] D. Porter, J. Voet, H. Bright, Mechanistic features of the D-amino acid oxidase reaction studied by double stopped flow spectrophotometry. J. Biol. Chem. 252 (1977) 4464-4473.

[56] A.K. Tan, R.R. Ramsay, Substrate-specific enhancement of the oxidative half-reaction of monoamine oxidase, Biochemistry (N.Y.). 32 (1993) 2137-2143.

[57] J.P. Roth, R. Wincek, G. Nodet, D.E. Edmondson, W.S. McIntire, J.P. Klinman, Oxygen isotope effects on electron transfer to O_2 probed using chemically modified flavins bound to glucose oxidase, J. Am. Chem. Soc. 126 (2004) 15120-15131.

[58] J.P. Roth, J.P. Klinman, Catalysis of electron transfer during activation of O2 by the flavoprotein glucose oxidase, PNAS. 100 (2003) 62-67.

[59] H. Hecht, H. Kalisz, J. Hendle, R. Schmid, D. Schomburg, Crystal Structure of Glucose Oxidase from *Aspergillus niger* Refined at 2.3 Å Reslution, J. Mol. Biol. 229 (1993) 153-172.

[60] I.J. Kass, N.S. Sampson, Evaluation of the role of His447 in the reaction catalyzed by cholesterol oxidase, Biochemistry (N.Y.). 37 (1998) 17990-18000.

[61] H. Mizutani, I. Miyahara, K. Hirotsu, et al., Three-dimensional structure of the purple intermediate of porcine kidney D-amino acid oxidase. Optimization of the oxidative half-reaction through alignment of the product with reduced flavin, J. Biochem. 128 (2000) 73-81.

[62] R. Baron, C. Riley, P. Chenprakhon, et al., Multiple pathways guide oxygen diffusion into flavoenzyme active sites, PNAS. 106 (2009) 10603-10608.

CHAPTER 8

EXPLORING THE BIOCATALYTIC SCOPE OF A BACTERIAL FLAVIN-CONTAINING MONOOXYGENASE

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ABSTRACT

A bacterial flavin-containing monooxygenase (FMO), fused to phosphite dehydrogenase, has been used to explore its biocatalytic potential. The bifunctional biocatalyst could be expressed in high amounts in *Escherichia coli* and was able to oxidize indole and indole derivatives into a variety of indigo compounds. The monooxygenase also performs the sulfoxidation of a wide range of prochiral sulfides, showing moderate to good enantioselectivities in forming chiral sulfoxides.

INTRODUCTION

Flavin containing monooxygenases (FMO) (E.C. 1.14.13.8) represent single-component flavoprotein monooxygenases that contain a tightly bound FAD cofactor [1]. In their catalytic cycle, the flavin cofactor is reduced by NADPH after which it reacts with molecular oxygen to form a hydroperoxyflavin intermediate [2–5]. This reactive enzyme species is able to perform oxygenation of heteroatom-containing compounds that are able to reach its active site. In humans, five FMO isoforms are present that show a tissue-specific distribution (see some recent examples:[6–8]). The role of these oxidative enzymes is thought to be similar to that of most human cytochrome P450 monooxygenases: detoxification of drugs and other xenobiotics into more hydrophilic metabolites [9,10]. The crucial detoxifying role of human FMOs becomes apparent when considering trimethylaminuria, also known as fish-odour syndrome. This metabolic disorder is caused by mutations in human FMO3 and results in accumulation of trimethylamine, causing a "fish odour" [11].

Most of the known FMOs have been shown to be membrane-associated, prohibiting facile isolation. As a result, FMOs have rarely been studied for biocatalytic purposes (see for example: [12–14]). Some years ago the first bacterial FMO, mFMO from *Methylophaga* sp. Strain SK1, was reported which was shown to be a soluble enzyme that could be overexpressed in *E. coli* [15]. In fact, inspection of sequenced bacterial genomes with the aid of an FMO-specific sequence motif has revealed a large number of genes that putatively encode soluble FMOs [16]. Therefore it is attractive to start exploring this class of monooxygenases.

Little biocatalytic data on mFMO have been reported. mFMO is a homodimer with subunits of 54 kDa, showing around 30% sequence identity with the five human FMOs [15]. mFMO was shown to be able to convert endogenous indole in *E. coli* cells and this capacity has been optimized to form up to 920 mg indigo blue per litre of fermentation broth [17]. It was also shown that the bacterial enzyme is able to convert similar substrates when compared with human FMOs, *e.g.* trimethylamine and (*S*)-(-)-nicotine are efficiently oxidized by mFMO.

Recently we have described the preparation of selfsufficient monooxygenases by covalent coupling of Baeyer–Villiger monooxygenases (BVMOs) with the soluble NADPH regenerating phosphite dehydrogenase (PTDH) from *Pseudomonas stutzeri* [18]. These bifunctional biocatalysts are able to use phosphite as a cheap and sacrificial substrate for recycling NADPH. An improved expression vector for producing these

bifunctional biocatalysts has been developed using a codon-optimized gene encoding a His-tagged and thermostable PTDH mutant as a fusion partner [19].

For this exploratory biocatalytic study on mFMO, we have used this newly developed expression vector. The produced selfsufficient mFMO was explored for the preparation of chiral sulfoxides, target molecules with a high interest due to their biological properties and widespread applications in organic synthesis [20–23]. Furthermore, we looked into the applicability of mFMO for the preparation of indigo derivatives, valuable as dyes or precursors for pharmaceuticals [24,25], which have been targeted before with other monooxygenases with varying degrees of success (see for example: [26–29]).

EXPERIMENTAL

Recombinant PTDH-mFMO was overexpressed and purified following the previously described procedure [18]. 1.0 Unit of PTDH-mFMO will oxidize 1.0 μmol of thioanisole to methyl phenyl sulfoxide per minute at pH 9.0 and room temperature in the presence of NADPH. Starting prochiral sulfides **1a**, **6a**, **8a** were purchased from Sigma–Aldrich–Fluka, **2–5a**, **7a**, **9–10a**, **11a**, **16a**, **17a**, **19a**, **20a** and **21a** were supplied by Alfa Aesar, **13a**, **22a**, **23a** and **24a** were obtained from Acros Organics and compound **25a** was a product by TCI Europe. All the starting indoles were products from Sigma-Aldrich-Fluka, with the exception of 4-chloroindole, which was purchased from AcrosOrganics. All other reagents and solvents were of the highest quality grade available.

Chemical reactions were monitored by analytical thin layer chromatography (TLC), performed on silica gel 60 F254 plates and visualized by UV irradiation. Flash chromatography was carried out with silica gel 60 (230–240 mesh). Kinetic parameters were measured in a Varian Cary50BioUV/Vis spectrophotometer. Melting points were taken on samples in open capillary tubes and are uncorrected. IR spectra were recorded on infrared spectrophotometer using KBr pellets. ^{1}H-NMR, ^{13}C-NMR and DEPT spectra were recorded with tetramethylsilane (TMS) as the internal standard with aDPX (^{1}H: 300.13 MHz; 13C: 75.5MHz) spectrometer. The chemical shift values (δ) are given in ppm. Optical rotations were measured using a polarimeter and are quoted in units of 10^{-1} deg cm^2 g^{-1}. APCI$^+$ and ESI$^+$ using a chromatograph mass detector or EI$^+$ with a mass spectrometer were used to record mass spectra (MS). High-resolution mass spectra were obtained with a BrukerMicrotof-Qspectrometer.

Methyl phenylethyl sulfide **15a** was synthesized by treating at 0 °C the corresponding thiol with sodium and methyl iodide in dry MeOH, under a nitrogen atmosphere (40% yield) [30]. 2- (Methylthio)pyridine **18a** was prepared by reaction of the corresponding thiol and potassium carbonate with iodomethane and triethylamine in CH_2Cl_2 at 0 °C (99% yield) [31]. *p*-Acetoxyphenyl methyl sulfide **2c** was obtained from **2a** by acetylation in presence of acetic anhydride and pyridine (90% yield). Racemic sulfoxides (±)-**1–15b**, (±)-**18–21b** and (±)-**23–24b** were prepared by chemical oxidation of the corresponding sulfides employing hydrogen peroxide and methanol (yields higher than 60%). All the synthesised compounds **15a** [32], **18a** [31], **1b** [30], **2b** [33], **3–7b** [30], **8b** [34], **9–15b** [30], **18b** [35], **19b** [36], **20b** [37], **21b** [35], **23b** [38], **24b** [35] exhibited physical and spectral data in agreement with those reported.

Absolute configurations of sulfoxides **1b** [30], **3–7b** [30], **9–15b** [30], **18b** [39], **19b** [40], **20b** [39], **21b** [36] were determined by comparison of elution order on HPLC with published data, meanwhile the absolute configurations of sulfoxide **2b** and **8b** were established by comparing the retention times on HPLC for acetylated **2b** and **8b** with the ones obtained in the asymmetric sulfoxidations of prochiral sulfides **2c** and **8a** employing (+)-diethyl L-tartrate, $Ti(O\text{-}i\text{-}Pr)_4$ and TBHP. **21** The absolute configuration of compound **23b** was obtained by comparing the specific rotation value with published data [41], while absolute configuration of butyl ethyl sulfoxide **24b** was established by analogy with this latest one.

General procedure for the oxidation of indole and derivatives employing PTDH-mFMO

A plate with 24 wells was employed. A solution of the corresponding starting indoles (5.0 mM) was dissolved in Tris-HCl buffer 50 mM, 35 mM NaCl, pH 8.5 containing sodium phosphite (10 mM), NADPH (0.2 mM) and self-sufficient biocatalyst PTDH-mFMO (4 mM). Reactions were stirred at 25 °C for 18 h and products formation was observed colorimetrically.

General procedure for the PTDH-mFMO biocatalysed sulfoxidations

Unless otherwise stated, prochiral sulfides **1–25a** (5 mM) were dissolved in a 50 mM Tris-HCl buffer pH 9.0 (1.0 mL) containing 1% DMSO, sodium phosphite

(10 mM), NADPH (0.2 mM) and the self-sufficient biocatalyst PTDH-mFMO (4 mM). Reactions were stirred at 25 °C and 250 rpm for the times established. Reactions were then stopped, extracted with EtOAc (2 x 0.5 mL), dried onto $MgSO_4$ and analysed by GC and/or HPLC in order to determine the conversions and the enantiomeric excesses of the sulfoxides (*R*)- or (*S*)-**1–25b**. Control reactions in absence of biocatalyst were performed and did not result in any conversion.

RESULTS AND DISCUSSION

The fused PTDH-mFMO could be readily expressed as soluble and bifunctional enzyme at high levels. By a one-step purification, 102 mg of pure and soluble PTDH-mFMO could be obtained from 1.0 L of culture broth. The bifunctional biocatalyst was tested for activity at several temperatures with trimethylamine. This revealed that the optimal temperature for activity is 70 °C. However, at this temperature the enzyme is quickly inactivated with a total loss of activity after 2 min. Analysis of the thermostability at 35 °C revealed a half-life of 5 h for mFMO, without affecting the activity of PTDH. For further experiments, it was decided to perform conversions at 25 °C. It was also established that the enzyme was most effective at pH 9.0 (see Supporting Information).

It is known that mFMO is able to form indigo blue from indole [15]. This was also clearly visible when growing cells for PTDH-mFMO overproduction: the growth medium turned dark blue. To establish whether mFMO can also be used for synthesis of other indigo derivatives, isolated PTDH-mFMO was incubated with a range of indole derivatives. Visual inspection revealed that all tested indoles were accepted as substrate as evidenced by formation of a variety of colours (Fig. 8.1). By steady-state kinetic analysis the reaction rates for all indole derivatives were determined (see Supporting Information). Indole was found to be converted most efficiently.

As mFMO has been shown to convert indoles, a set of substituted aromatic sulfides was tested as substrates. Most of the tested sulfides were shown to be oxidized and exhibited quite good K_M values, ranging from 100 to 600 mM, while the k_{cat} values were only modest: ~0.5 s^{-1} (see Supporting Information). PTDH-mFMO was subsequently assayed for enantioselective oxidation of thioanisole derivatives (Table 8.1). Reactions were performed in Tris-HCl buffer which contained 1% DMSO in order to dissolve the sulfides. In most cases, the (*S*)-sulfoxides were formed in excess.

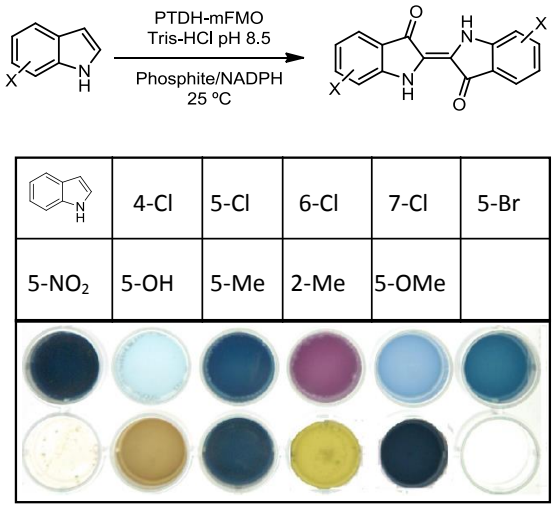
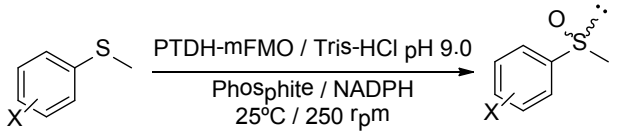


Figure 8.1. PTDH-mFMO catalysed oxidation of indole and indole derivatives into indigoid dyes.

Table 8.1. Sulfoxidation of thioanisole derivatives catalysed by the fused PTDH-mFMO biocatalyst.[a]



1-10a (*R*)- or (*S*)-**1-10b**

Sulfide	X	time [h]	*c* [%][b]	*e.e.* [%][c]
1a	H	8	95	35 (*S*)
2a	*p*-OH	15	37	81 (*S*)
3a	*p*-OMe	15	78	70 (*S*)
4a	*p*-Me	15	66	92 (*S*)
5a	*p*-Cl	15	80	95 (*S*)
6a	*m*-Cl	15	69	15 (*R*)
7a	*o*-Cl	15	81	75 (*R*)
8a	*p*-COMe	15	8	21 (*R*)
9a	*p*-CN	15	50	22 (*S*)
10a	*p*-NO_2	15	47	37 (*S*)

[a] For reaction details, see Experimental Section.

[b] Determined by GC.

[c] Optical purities determined by HPLC.

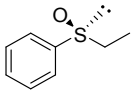
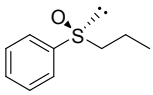
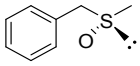
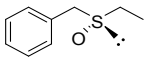
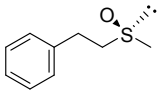
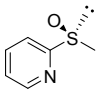
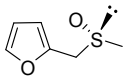
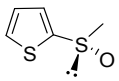
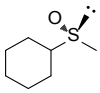
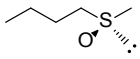
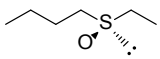
The most effective conversion was found with thioanisole **1a**, yielding 95% of (*S*)-methyl phenyl sulfoxide **1b** after 8 h, albeit with a moderate enantiomeric excess (*e.e.* = 35%). Substituents in the aromatic ring decreased the enzymatic activity. PTDH-mFMO was able to oxidize methyl *p*-tolyl sulfide (**4a**) and *p*-chlorophenyl methyl sulfide (**5a**) into the corresponding sulfoxides (*S*)-**4–5b** with high enantiomeric excesses (*e.e.* >90%). Good enantioselectivities were also obtained in the oxidation of the *p*-hydroxy (**2a**), *p*-methoxy (**3a**) and *o*-chloro (**7a**) derivatives, while the chlorosubstitution in the *meta*-position (**6a**) as well as the presence of electron-withdrawing groups (**8–10a**) had a negative effect on the selectivity of the formed sulfoxide.

As well as oxidation of thioanisole derivatives, some other aromatic sulfides were also tested. As indicated in Table 8.2, the oxidation of phenyl ethyl sulfide (**11a**) and its propyl analogue (**12a**) led to a significant loss in conversion when compared to thioanisole, but the corresponding (*S*)-sulfoxides can be obtained with much better enantiomeric excess (*e.e.* = 79%). (*S*)-Benzyl methyl sulfoxide (*S*)-**13b** and the ethyl derivative **14b** were formed with excellent or good conversions, respectively, while the biocatalyst showed a poor enantioselectivity for these compounds. When the sulfur atom was further away from the phenyl moiety (**15a**) a good enzymatic activity and moderate enantioselectivity was observed. No oxidation was achieved for those sulfides presenting one (methyl naphthyl sulfide, **16a**) or two bulky substituents (benzyl phenyl sulfide, **17a**). PTDH-mFMO has also been tested in the synthesis of heteroaromatic chiral sulfoxides, important chiral auxiliaries in asymmetric catalysis. Methyl 2-pyridyl sulfide, 2-furfurylmethyl methyl sulfide and 2-(methylthio)thiophene were converted into the corresponding sulfoxides with high conversions (around 80%). (*S*)-**18b** and (*S*)-**20b** were obtained with moderate enantiomeric excess, while the formation of (*R*)-**l9b** occurred with a low selectivity.

As mFMO has been shown to be active on aliphatic amines, PTDH-mFMO was also tested with several aliphatic sulfides. This has revealed that it displays a relatively low activity and selectivity for the oxidation of cycloalkyl alkyl sulfides (**21a**). Sulfoxidation of tetrahydro-2*H*-thiopyran (**22a**) did not take place, even after long reaction times. Finally, we focused on the biooxidation of linear aliphatic sulfides. It was found that alkyl butyl sulfides **23a** and **24a** were oxidized with moderate conversion and good enantiomeric excesses in order to obtain (*R*)-**23b** and (*R*)-**24b**, respectively. The presence of a longer alkyl chain in the

sulfide structure had a negative effect on the reactivity, as no reaction was observed in the oxidation of *n*-octyl methyl sulfide **25a**.

Table 8.2. PTDH-mFMO catalysed synthesis of chiral sulfoxides starting from the corresponding sulfide.[a]

Sulfide	Sulfoxide	t [h]	*c* [%][b]	*e.e.* [%][c]
11a		15	15	79 (*S*)
12a		24	12	71 (*S*)
13a		8	91	17 (*S*)
14a		8	75	15 (*S*)
15a		15	80	36 (*S*)
18a		15	83	49 (*S*)
19a		15	72	20 (*R*)
20a		15	76	50 (*S*)
21a		15	14	20 (*R*)
23a		15	52	85 (*R*)
24a		15	71	77 (*R*)

[a] For reaction details, see Experimental Section.

[b] Determined by GC.

[c] Optical purities determined by HPLC.

PTDH-mFMO was also tested in the oxidation of racemic sulfoxides. Incubating racemic methyl phenyl sulfoxide (±)-**1b** with this biocatalyst led to a moderate conversion, with only 8% of the final sulfone while **1b** remained racemic. No sulfoxide oxidation was observed for compounds (±)-**4b**, (±)-**5b** and (±)-**7b** after long reaction times.

The close homology of FMOs and BVMOs [1] inspired us to test substrates that are readily oxidized by the latter type of enzymes. However, after long reaction times, no product formation was observed in the presence of different ketones (2-octanone, cyclohexanone and acetophenone) or phenylboronic acid.

In a previous report, the influence of the organic cosolvents on the oxidation of prochiral sulfides catalysed by BVMOs has been described [42]. Short alkyl chain alcohols as cosolvents were shown to result in an increase or even a reversal in enantioselectivity for some BVMOs. To probe this effect on mFMO, oxidation of **1a** was performed in the presence of several organic cosolvents (5% v/v) with different physico-chemical properties. All the cosolvents tested led to lower conversions and/or enantiomeric excesses (see Supporting Information), with the exception of the reaction in 5% hexane. For this cosolvent, (*S*)-**1b** was recovered with a 48% *e.e.* and 62% extent, while 28% of methyl phenyl sulfone **1c** was formed. Sulfoxidation with a combination of 1% DMSO and 5% hexane occurred only with 24% conversion and 34% *e.e.* for (*S*)-**1b**. From the data it becomes clear that 5% hexane has an activating effect on the oxidation of the sulfoxide **1b**, while the presence of DMSO in the reaction medium inhibits sulfoxide oxidation, as can be shown in the experiments developed by employing PTDH-mFMO in the biooxidation of (±)-**1b** (entries 6–8, Table 8.3). Thus, the increase in the enantiomeric excess observed when working in 5% hexane is due to the desymmetrization of prochiral **1a** combined with the kinetic resolution of **1b**. *n*-Hexane presents a somewhat similar structure to 1-octylamine, which has been described as a specific activator in human FMOs (see for example: [43,44]). The presence of 3 mM of this additive led to a higher conversion while it did not significantly affect the enantioselectivity (see entry 5).

Table 8.3. Effect of cosolvent in the sulfoxidation of **1a**.[a]

Entry	Sulf.	Cosolvent	*c* [%][b]	*e.e.* (*S*)-**1b** [%][c]
1	1a	None	48 (2)	33
2	1a	1% DMSO	95 (5)	35
3	1a	5% Hexane 1% DMSO	90 (28)	48
4	1a	5% Hexane	24	34
5	1a	3 mM $OctNH_2$	76 (7)	27
6	(±)-**1b**	1% DMSO	8	5
7	(±)-**1b**	5% Hexane	61	72
8	(±)-**1b**	5% Hexane 1% DMSO	9	5

[a] Reaction time: 8 h. For other reaction details, see Experimental Section.

[b] Determined by GC. In brackets, the amount of sulfone formed.

In order to show that this bifunctional enzyme can be used as an effective biocatalyst, we set up a semipreparative experiment, in which sulfide **5a** was used as model substrate. Sulfoxidation was performed by incubating 100 mg of prochiral **5a** in the presence of PTDH-mFMO (4.0 mM), sodium phosphite as cosubstrate, NADPH (0.2 mM) and dioxygen as oxidant. After 24 h, (*S*)-**5b** was achieved with a 90% conversion and 95% enantiomeric excess. The sulfoxide product was isolated by column chromatography, to afford 77% isolated yield.

CONCLUSIONS

A novel fused oxidative biocatalyst has been overexpressed in *E. coli*, isolated and purified. PTDH-mFMO was found to be able to oxidize indole and analogues into the corresponding indigoid pigments which represent interesting dyes and bioactive compounds. The bifunctional dehydrogenase-FMO system has been applied for the first time in the biocatalysed sulfoxidation of prochiral sulfides. Depending on the substrate structure, excellent enantioselectivities can be achieved. The data show that bacterial FMOs represent an unexplored and interesting class of oxidative biocatalysts which may be of use for synthetic purposes. The availability of the crystal structure of mFMO [2] can also facilitate redesign of FMOs for specific biocatalytic applications.

SUPPORTING INFORMATION

Experimental procedures

Asymmetric oxidation of sulfide 8a employing the Kagan methodology [45]

Ti(O-*i*-Pr)$_4$ (10 mmoles) and (*R*,*R*)-DET (+)-diethyl L-tartrate (20 mmoles) were dissolved at room temperature in 50 mL of CH_2Cl_2 under nitrogen atmosphere. Distilled water (5 mmoles) was then added dropwise. Stirring was maintained until the yellow solution formed became homogeneous and sulfide **8a** was added (5 mmoles). The resulting mixture was cooled to – 20 °C and a 5.5 M TBHP (*tert*-butyl hydroperoxide) solution in decane (5.5 mmoles) was added. After four hours, additional water was added dropwise (50 mmoles). A strong stirring was maintained for 1 hour at – 20 °C and then for one additional hour at room temperature.The gel obtained was filtrated and washed with CH_2Cl_2 (10 mL). The filtrate was kept in the presence of a mixture of NaOH (5%) in brine (30 mL) for 1 hour and then extracted with CH_2Cl_2 (3×20 mL). The organic phase was dried over Na_2SO_4 and concentrated to give the crude product, which was purified by flash chromatography using a mixture of CH_2Cl_2/MeOH (95:5) as eluent in order to give 4-acetylphenyl methyl sulfoxide (*R*)-**8b**.

(*R*)-4-acetylphenyl methyl sulfoxide, (*R*)-8b: Yield 40% (344 mg)**.** $[\alpha]_D^{25}$= +90.2 (*c* 1.0, acetone), *e.e.* = 87%.

Asymmetric oxidation of sulfide 2a employing the Kagan methodology

p-Hydroxyphenyl methyl sulfide **2a** was acylated with acetic anhydride and pyridine in order to obtain *p*-acetoxyphenyl methyl sulfide **2c** (90% yield). This substrate was oxidised by the methodology previously described except for an acidification step with HCl 1.0 M before the extraction with CH_2Cl_2, obtaining (*R*)- *p*-acetoxyphenyl methyl sulfoxide in 16% yield and *e.e.* = 30% after purification by flash chromatography using a mixture of CH_2Cl_2 / MeOH (95:5) as eluent.

***p*-Acetoxyphenyl methyl sulfide (2c)**. (90% yield). White solid. Mp: 43-44 °C IR (KBr): ν 2924, 1760, 1489, 1370, 1202, 739 cm^{-1}. ^{1}H-NMR (300.13 MHz, $CDCl_3$, 25 °C): δ 2.21 (s, 3H), 2.39 (s, 3H), 6.97 (d, $^3J_{H,H}$ 6.8 Hz, 2H), 7.21 (d, $^3J_{H,H}$ 6.8 Hz, 2H). ^{13}C-NMR (75.5 MHz, $CDCl_3$, 25 °C): δ 15.6 (CH_3), 20.4 (CH_3), 121.6 ($2CH_{ar}$), 127.2 ($2CH_{ar}$), 135.2 (C_{ar}), 147.9 (C_{ar}), 168.8

(CO). (EI$^+$, *m/z*): 182 (M^+, 41%), 140 (100), 125 (44), 96 (13), 43 (33). HRMS (ESI$^+$): calcd. for $C_9H_{10}NaO_2S$ $(M+Na)^+$: 205.0295; found: 205.0294.

Biocatalysed sulfoxidation of thioanisole at different pHs

Thioanisole (**1a**) (5 mM) was dissolved in a Tris-HCl 50 mM buffer (pH from 6.0 to 10.0) containing sodium phosphite (10 mM), NADPH (0.2 mM) and the self-sufficient biocatalyst PTDH-mFMO (4 μM). Reactions were stirred at 25 °C and 250 rpm for 8 h. Crude mixtures were then stopped, extracted with EtOAc (2 x 0.5 mL), dried onto $MgSO_4$ and analysed by GC and HPLC in order to determine the conversions and the optical purities of (*S*)-**1b**, as shown in Figure S8.1. Control reactions in absence of biocatalyst were performed for all substrates, not observing formation of the final products.

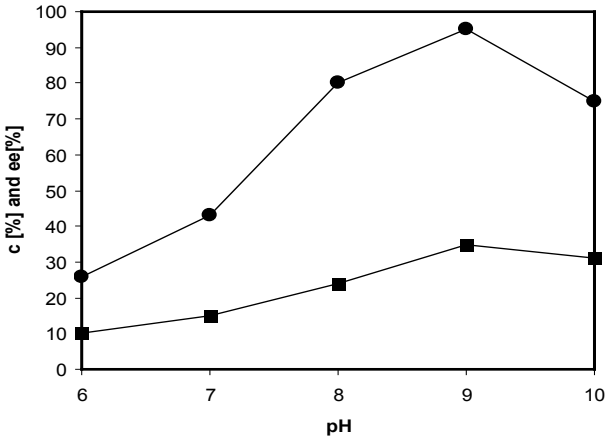


Figure S8.1. Effect of pH in the conversion (●) and in the enantiomeric excess (■) for the PTDH-mFMO biocatalysed oxidation of thioanisole **1a**. The values represent an average of at least 3 measurements with standard errors less than 5%.

Biocatalysed sulfoxidation of thioanisole in presence of organic cosolvents

Thioanisole (**1a**) (5 mM) was dissolved in a Tris-HCl 50 mM buffer pH 9.0 containing a 5% v/v of organic cosolvent (1.0 mL), sodium phosphite (10 mM), NADPH (0.2 mM) and the self-sufficient biocatalyst PTDH-mFMO (4 μM). Reactions were

stirred at 25 °C and 250 rpm for 8 h. Crude mixtures were then stopped, extracted with EtOAc (2 x 0.5 mL), dried onto $MgSO_4$ and analysed by GC and HPLC in order to determine the conversions and the optical purities of (*S*)-**1b**. Control reactions in absence of biocatalyst were performed for all substrates, not observing formation of the final products.

PTDH-mFMO catalysed oxidation of racemic sulfoxides (±)-1b, (±)-4-5b and (±)-7b

Sulfoxides (±)-**1b**, (±)-**4-5b** or (±)-**7b** (5 mM) were dissolved in a Tris-HCl buffer pH 9.0 containing 1% DMSO and the organic cosolvent when stated (final volume 1.0 mL), sodium phosphite (10 mM), NADPH (0.2 mM) and the self-sufficient biocatalyst PTDH-mFMO (4 μM). Reactions were stirred at 25 °C and 250 rpm for the times established. Reactions were then stopped, extracted with EtOAc (2 x 0.5 mL), dried onto MgSO4 and analysed by GC and/or HPLC in order to determine the conversions and the optical purities of the remaining sulfoxides. Control reactions in absence of biocatalyst were performed for all substrates, not observing formation of the final products.

Table S8.1. Effect of organic cosolvents in the biocatalysed oxidation of thioanisole employing PTDH-mFMO.

Entry	Cosolvent	*c* [%][a]	*e.e.* [%][b]
1	None	48	33
2	1% DMSO	95	35
3	5% DMSO	21	29
4	5% DMF	22	29
5	5% MeOH	45	18
6	5% 1,4-dioxane	≤ 3	n.d.
7	5% *i*PrOH	22	18
8	5% *t*BuOMe	12	15
9	5% Toluene	9	26
10	5% 2-octanol	45	36

[a] Measured by GC. [b] Determined by HPLC. n.d. not determined

Kinetic data

PTDH-mFMO concentration were measured photometrically by monitoring the absorption of the FAD-cofactor at 450 nm (ε_{450} = 11.3 mM^{-1} cm^{-1}) after treating the enzyme with 0.1% SDS.

Steady-state kinetics

The enzymatic activity of PTDH-mFMO for the oxidation of indole and its derivatives, trimethylamine, sulfides **1a**, **3a**, **5a**, **9a**, **13a**, **21a** and **24a**, coenzymes NADPH and NADP+ and sodium phosphite were determined spectrophotometrically by monitoring NADPH consumption or formation at 340 nm ($\varepsilon_{340} = 6.22\ mM^{-1}cm^{-1}$).

Stock solutions of sulfides **1a**, **3a**, **5a**, **9a**, **13a**, **21a** and **24a** (250 mM) and indoles (1.0 M) were made in 1,4-dioxane meanwhile 10 mM $NADP^+$, NADPH, sodium phosphite and trimethylamine (500 mM) stock solutions were prepared in Tris-HCl (50 mM, 35 mM NaCl, pH 8.5) buffer. A reaction mixture (1.0 mL) usually contained Tris-HCl (25 mM, 35 mM NaCl, pH 9.0 for the sulfides and pH 8.5 for indoles, trimethylamine, NADPH and NADP+), 100 μM NADPH, 1% v/v 1,4-dioxane for the measurements of the sulfides and indoles, and 0.17-1.00 μM PTDH-mFMO.

Table S8.2. Steady-state kinetic parameters for PTDH-mFMO.

Compound	K_M [μM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$M^{-1}s^{-1}$]
$NADP^+$	19±3	6.3±0.11	330,000
NADPH	11±1	5.0±0.03	460,000
Sodium phosphite	1600±25	3.6±0.02	2,200
Trimethylamine	8±1	4.7±0.10	590,000
Thioanisole	170±8	0.45±0.01	2,600
4-Chlorothioanisole	87±6	0.25±0.02	2,900
4-Methoxythioanisole	220±15	0.23±0.01	1,000
4-Cyanothioanisole	590±10	0.29±0.01	490
Benzyl methyl sulfide	470±12	0.34±0.01	720
Cyclohexyl methyl sulfide	350±13	0.19±0.01	540
Butyl ethyl sulfide	94±3	0.62±0.02	6,600

Table S8.3. Catalytic efficiencies in the PTDH-mFMO catalysed oxidation of indole and derivatives.

Compound	k_{obs} [s^{-1}][a]
Indole	0.090
4-Chloroindole	0.061
5-Chloroindole	0.070
6-Chloroindole	0.077
7-Chloroindole	0.063
5-Bromoindole	0.066
5-Nitroindole	0.035
5-Hydroxyindole	0.042
5-Methylindole	0.042
5-Methoxyindole	0.038
2-Methylindole	0.079

[a] For all compounds, standard deviations (average 4 measures) were below ±5.0%.

Table S8.4. Determination of conversion values and enantiomeric excesses by GC.

Substrate	Program[a]	Column	t_R [min] sulfides	t_R [min] sulfoxides
1a	70/10/190	B	3.2	5.9
2a	100/5/3/200	B	8.6	19.6
3a	70/10/190	B	6.1	9.3
4a	70/0/10/190	B	4.5	7.4
5a	70/0/10/190	B	5.5	8.2
6a	70/0/10/190	B	5.5	7.4
7a	70/0/10/190	B	5.5	5.5
8a	100/5/3/130	B	12.6	19.4
9a	70/0/10/190	B	7.4	9.4
10a	70/0/10/190	B	8.9	10.6
11a	70/0/10/190	B	3.8	7.1
12a	70/0/10/190	B	4.7	8.2
13a	70/0/10/190	B	4.1	7.6
14a	70/0/10/190	B	4.4	7.7
15a	70/5/3/150	B	12.7	24.5
18a	70/5/3/200	B	5.4	12.9
19a	70/0/10/190	B	2.4	5.2
20a	70/5/3/130	B	8.7	16.6
21a	70/5/3/150	B	4.6	16.4
23a	50/5/3/200	A	12.2	31.4 (*S*), 31.8 (*R*)
24a	50/5/3/200	A	14.2	33.1(*S*), 35.4 (*R*)

[a] Program: initial T (ºC)/ time (min)/ slope (ºC/min)/T (ºC)/ time (min).

GC and HPLC Analyses

The following columns were used for the determination of conversions and enantiomeric excesses of the sulfoxides by GC: A: Restek RT-BetaDEXse (30 m x 0.25 mm x 0.25 µm, 12 psi N_2) and B: Hewlett Packard HP-1 (30m x 0.32 mm x 0.25µm, 12.2 psi N_2).

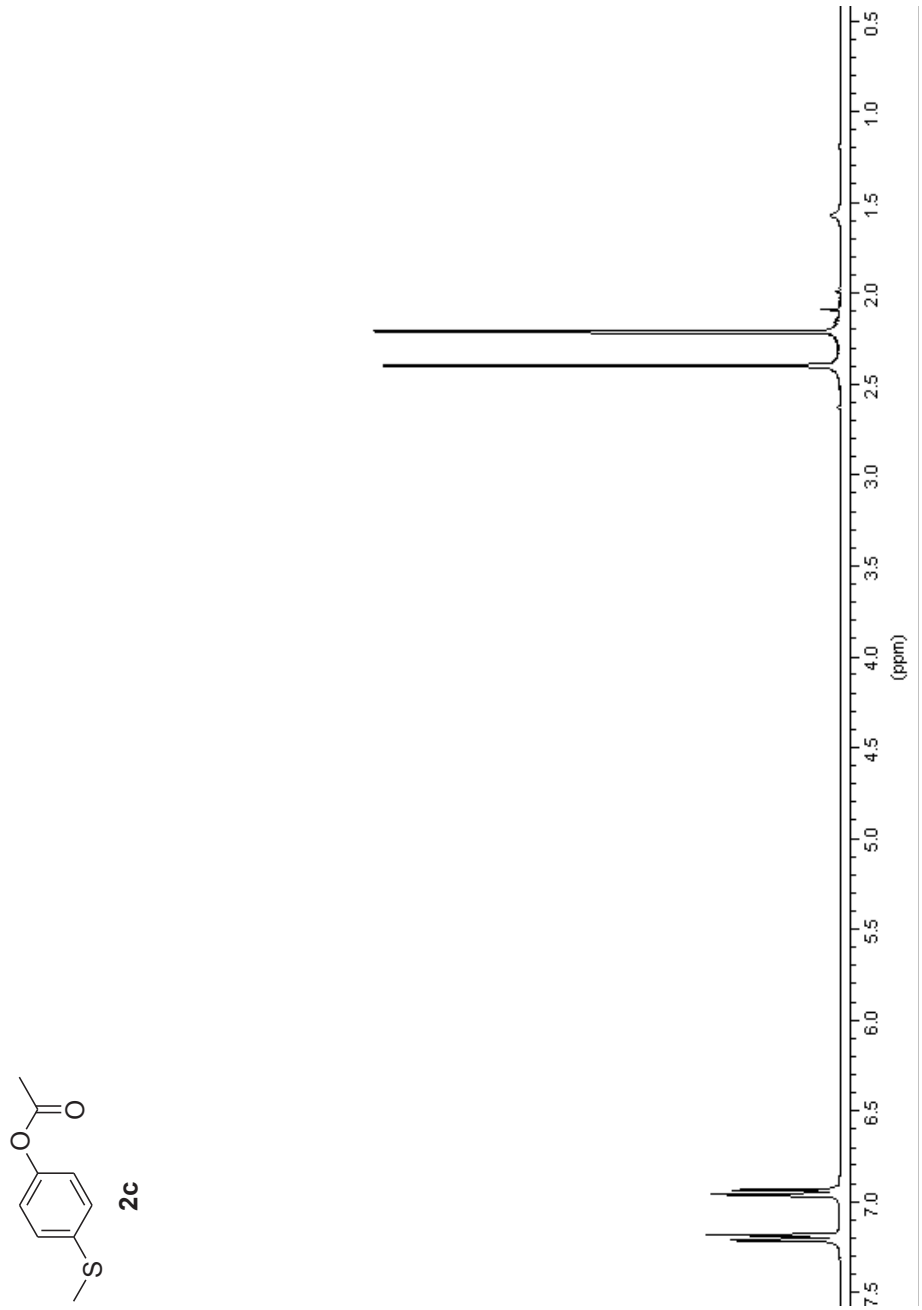
For the determination of the enantiomeric excesses of the sulfoxides by HPLC, the following columns were employed: column A: Chiralcel OB-H (0.46 cm x 25 cm), column B: Chiralcel OD (0.46 cm x 25 cm), column C: Chiralpak IA (0.46 cm x 25 cm), column D: Chiralcel OD-H (0.46 x 25 cm) and column E: Chiralcel OJ-H (0.45 cm x 25 cm), all of them are from Daicel.

Table S8.5. Determination of enantiomeric excesses by HPLC.

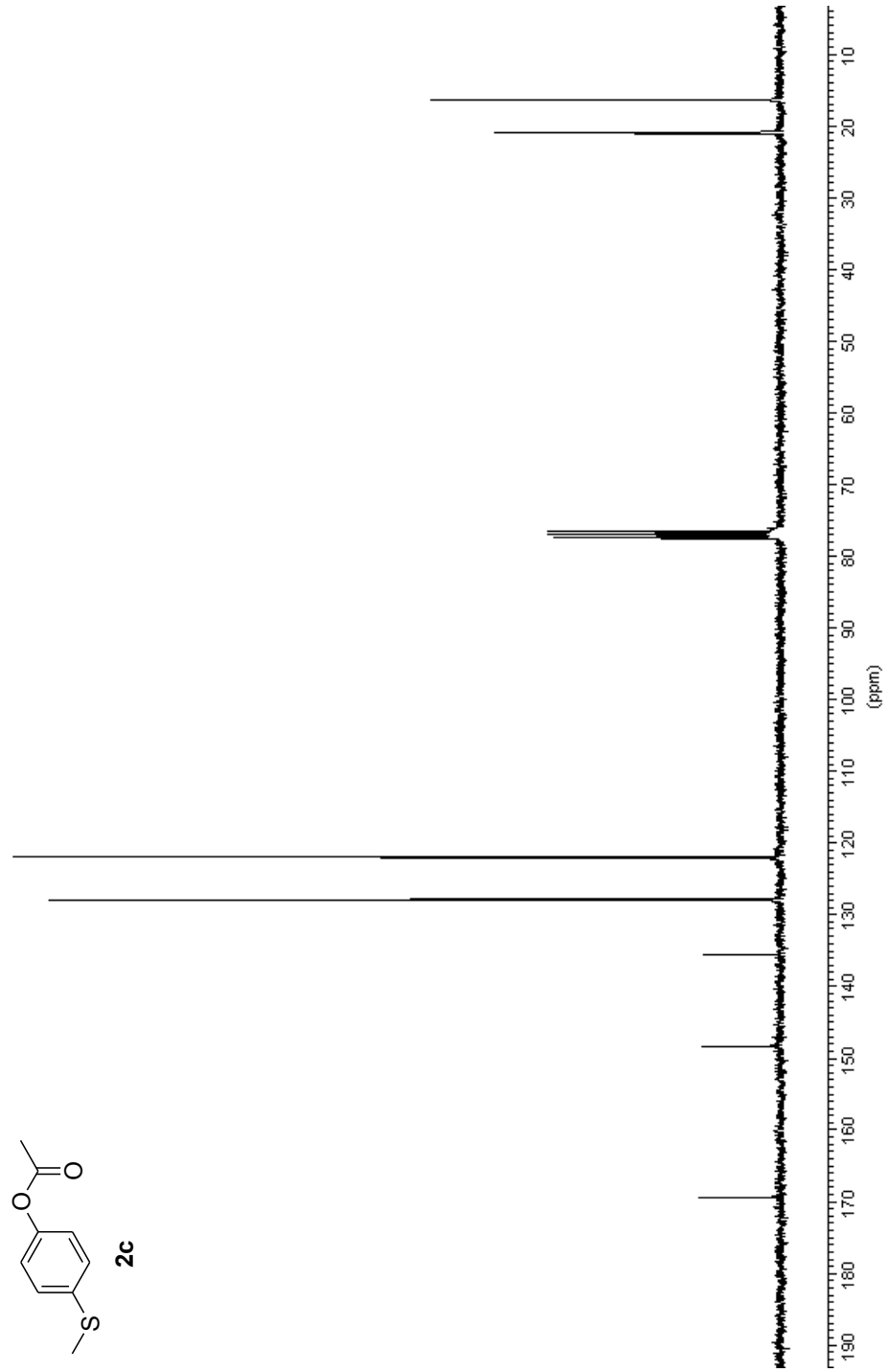
Substrate	Column	Flow rate [mL min^{-1}]	T [°C]	Eluent[a]	Retention time [min]
1b	B	0.8	25	*n*-hexane-IPA 9:1	13.6 (*R*); 16.8 (*S*)
2b[b]	C	0.7	20	*n*-hexane-EtOH 95:5[c]	38.7 (*R*); 39.9 (*S*)
3b	D	1.0	25	*n*-hexane-IPA 9:1	15.2 (*R*); 17.1 (*S*)
4b	A	0.5	20	*n*-hexane-IPA 9:1	20.6 (*R*); 22.9 (*S*)
5b	A	0.5	20	*n*-hexane-IPA 8:2	18.5 (*S*); 28.3 (*R*)
6b	A	0.8	30	*n*-hexane-IPA 8:2	10.8 (*R*); 14.6 (*S*)
7b	A	0.5	20	*n*-hexane-IPA 8:2	17.2 (*S*); 29.6(*R*)
8b	E	0.7	30	*n*-hexane-EtOH 8:2	21.9 (*S*); 23.5 (*R*)
9b	C	1.0	30	*n*-hexane-IPA 8:2	20.7 (*R*); 23.0 (*S*)
10b	A	1.0	25	*n*-hexane-IPA 8:2	39.1 (*S*); 50.0 (*R*)
11b	B	0.5	20	*n*-hexane-IPA 9:1	18.1 (*R*); 22.2 (*S*)
12b	A	1.0	25	*n*-hexane-IPA 8:2	13.5 (*S*); 16.7 (*R*)
13b	B	0.8	25	*n*-hexane-IPA 9:1	18.3 (*R*); 19.8 (*S*)
14b	B	1.0	25	*n*-hexane-IPA 9:1	17.7 (*R*); 19.1 (*S*)
15b	A	1.0	25	*n*-hexane-IPA 8:2	40.2 (*S*); 42.7 (*R*)
18b	A	1.0	25	*n*-hexane-IPA 9:1	16.6 (*S*); 29.8 (*R*)
19b	A	1.0	25	*n*-hexane-IPA 8:2	12.6 (*S*); 14.3 (*R*)
20b	A	0.5	25	*n*-hexane-IPA 92:8	21.4 (*S*); 26.5 (*R*)
21b	A	0.7	25	*n*-hexane-IPA 8:2	8.1 (*R*); 9.7 (*S*)

[a] Experiments were performed with isocratic eluent. [b] Compound **2b** was derivatised into the corresponding acetate, compound **2c**. [c] Starting flow 95:5 *n*-hexane-EtOH, increased polarity until 90:10 in 15 minutes and from 90:10 to 80:20 in 30 minutes.

NMR SPECTRA



2c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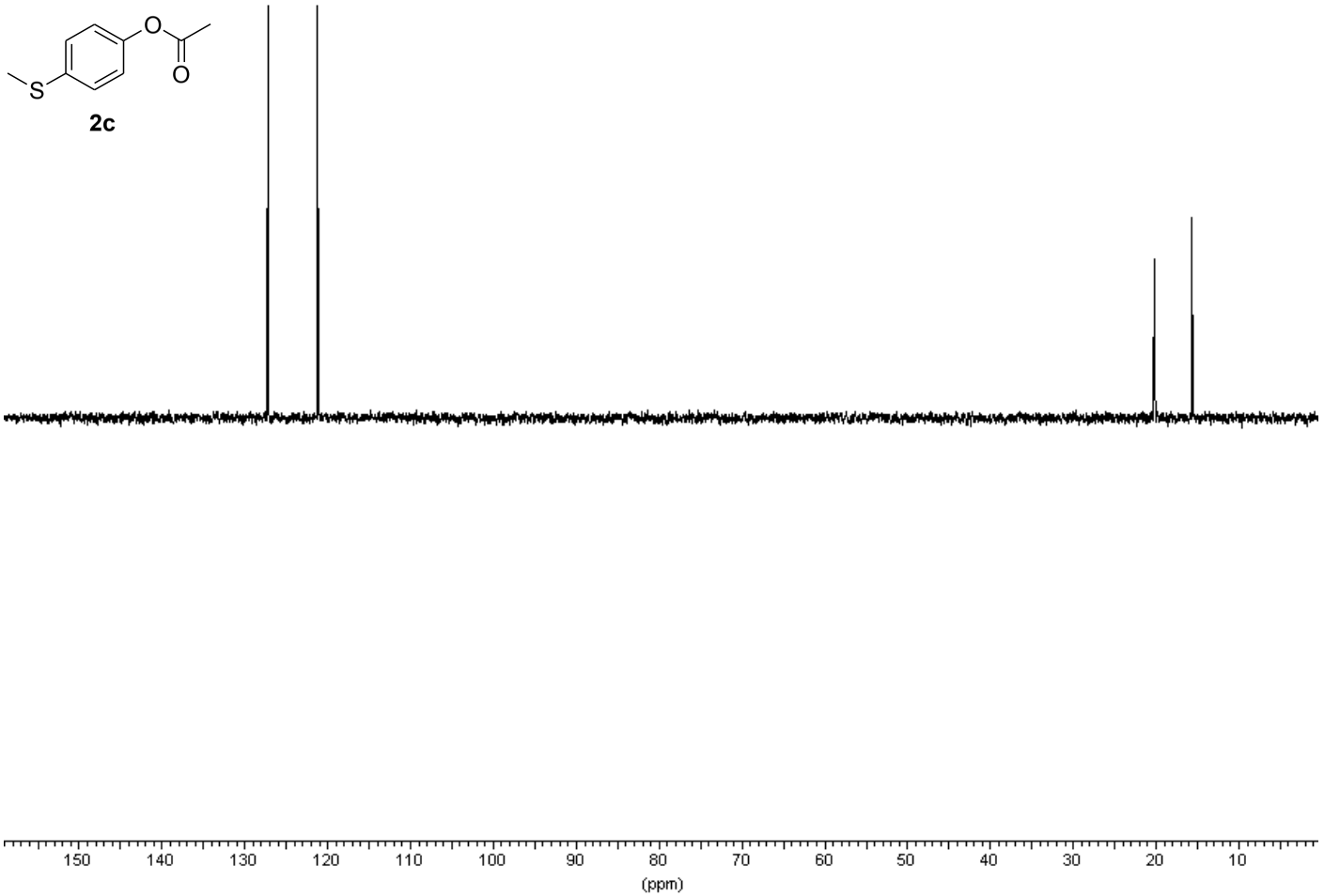
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REFERENCES

[1] W.J. van Berkel, N.M. Kamerbeek, M.W. Fraaije, Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts, J. Biotechnol. 124 (2006) 670–689.

[2] A. Alfieri, E. Malito, R. Orru, M.W. Fraaije, A. Mattevi, Revealing the moonlighting role of NADP in the structure of a flavin-containing monooxygenase, Proc. Natl. Acad. Sci. 105 (2008) 6572-6577.

[3] R. Orru, D.E.T. Pazmiño, M.W. Fraaije, A. Mattevi, Joint functions of protein residues and NADP(H) in oxygen activation by flavin-containing monooxygenase., J. Biol. Chem. 285 (2010) 35021–8.

[4] D.M. Ziegler, An overview of the mechanism, substrate specificities, and structure of FMOs., Drug Metab. Rev. 34 (2002) 503–511.

[5] J.R. Cashman, Structural and Catalytic Properties of the Mammalian Flavin-Containing Monooxygenase, Chem. Res. Toxicol. 8 (1995) 165–181.

[6] S.J. Sadeghi, R. Meirinhos, G. Catucci, V.R. Dodhia, G. Di Nardo, G. Gilardi, Direct electrochemistry of drug metabolizing human flavin-containing monooxygenase: electrochemical turnover of benzydamine and tamoxifen., J. Am. Chem. Soc. 132 (2010) 458–459.

[7] I.R. Phillips, E.A. Shephard, Flavin-containing monooxygenases: mutations, disease and drug response, Trends Pharmacol. Sci. 29 (2008) 294–301.

[8] J.R. Cashman, J. Zhang, Human flavin-containing monooxygenases., Annu. Rev. Pharmacol. Toxicol. 46 (2006) 65–100.

[9] J.R. Cashman, Some distinctions between flavin-containing and cytochrome P450 monooxygenases, Biochem. Biophys. Res. Commun. 338 (2005) 599–604.

[10] J.R. Cashman, The role of flavin-containing monooxygenases in drug metabolism and development., Curr. Opin. Drug Discov. Devel. 6 (2003) 486–493.

[11] J.R. Cashman, K. Camp, S.S. Fakharzadeh, P. V Fennessey, R.N. Hines, O.A. Mamer, et al., Biochemical and clinical aspects of the human flavin-containing monooxygenase form 3 (FMO3) related to trimethylaminuria., Curr. Drug Metab. 4 (2003) 151–170.

[12] R.J. Krause, S.C. Glocke, A.R. Sicuri, S.L. Ripp, A.A. Elfarra, Oxidative metabolism of seleno-L-methionine to L-methionine selenoxide by flavin-containing monooxygenases., Chem. Res. Toxicol. 19 (2006) 1643–1649.

[13] A.E. Rettie, M.P. Lawton, A.J. Sadeque, G.P. Meier, R.M. Philpot, Prochiral sulfoxidation as a probe for multiple forms of the microsomal flavin-containing monooxygenase: studies with rabbit FMO1, FMO2, FMO3, and FMO5 expressed in *Escherichia coli*., Arch. Biochem. Biophys. 311 (1994) 369–77.

[14] T. Nagata, D.E. Williams, D.M. Ziegler, Substrate specificities of rabbit lung and porcine liver flavin-containing monooxygenases: differences due to substrate size, Chem. Res. Toxicol. 3 (1990) 372–376.

[15] H.S. Choi, J.K. Kim, E.H. Cho, Y.C. Kim, J. Il Kim, S.W. Kim, A novel flavin-containing monooxygenase from *Methylophaga* sp. strain SK1 and its indigo synthesis in *Escherichia coli*, Biochem. Biophys. Res. Commun. 306 (2003) 930–936.

[16] M.W. Fraaije, N.M. Kamerbeek, W.J.H. Van Berkel, D.B. Janssen, Identification of a Baeyer-Villiger monooxygenase sequence motif, FEBS Lett. 518 (2002) 43–47.

[17] G.H. Han, H.J. Shin, S.W. Kim, Optimization of bio-indigo production by recombinant *E. coli* harboring fmo gene, Enzyme Microb. Technol. 42 (2008) 617–623.

[18] D.E. Torres Pazmiño, R. Snajdrova, B.-J. Baas, M. Ghobrial, M.D. Mihovilovic, M.W. Fraaije, Self-Sufficient Baeyer–Villiger Monooxygenases: Effective Coenzyme Regeneration for Biooxygenation by Fusion Engineering, Angew. Chemie. 120 (2008) 2307–2310.

[19] D.E. Torres Pazmiño, A. Riebel, J. de Lange, F. Rudroff, M.D. Mihovilovic, M.W. Fraaije, Efficient biooxidations catalyzed by a new generation of self-sufficient Baeyer-Villiger monooxygenases., ChemBioChem. 10 (2009) 2595–8.

[20] E. Wojaczyńska, J. Wojaczyński, Enantioselective synthesis of sulfoxides: 2000-2009., Chem. Rev. 110 (2010) 4303–4356.

[21] M.C. Carreño, G. Hernández-Torres, M. Ribagorda, A. Urbano, Enantiopure sulfoxides: recent applications in asymmetric synthesis., Chem. Commun. (Camb). (2009) 6129–6144.

[22] H. Pellissier, Use of chiral sulfoxides in asymmetric synthesis, Tetrahedron. 62 (2006) 5559–5601.

[23] R. Bentley, Role of sulfur chirality in the chemical processes of biology., Chem. Soc. Rev. 34 (2005) 609–624.

[24] K. McClay, C. Boss, I. Keresztes, R.J. Steffan, Mutations of toluene-4-monooxygenase that alter regiospecificity of indole oxidation and lead to production of novel indigoid pigments., Appl. Environ. Microbiol. 71 (2005) 5476–5483.

[25] F.P. Guengerich, J.L. Sorrells, S. Schmitt, J.A. Krauser, P. Aryal, L. Meijer, Generation of new protein kinase inhibitors utilizing cytochrome p450 mutant enzymes for indigoid synthesis., J. Med. Chem. 47 (2004) 3236–3241.

[26] N.N. Rosic, Versatile capacity of shuffled cytochrome P450s for dye production., Appl. Microbiol. Biotechnol. 82 (2009) 203–210.

[27] E.W. van Hellemond, D.B. Janssen, M.W. Fraaije, Discovery of a novel styrene monooxygenase originating from the metagenome., Appl. Environ. Microbiol. 73 (2007) 5832–5839.

[28] A. Celik, R.E. Speight, N.J. Turner, Identification of broad specificity P450CAM variants by primary screening against indole as substrate., Chem. Commun. (Camb). (2005) 3652–3654.

[29] Q.S. Li, U. Schwaneberg, P. Fischer, R.D. Schmid, Directed evolution of the fatty-acid hydroxylase P450 BM-3 into an indole-hydroxylating catalyst., Chemistry. 6 (2000) 1531–6.

[30] G. De, D.E. Torres Pazmiño, G. Ottolina, M.W. Fraaije, G. Carrea, 4-Hydroxyacetophenone monooxygenase from *Pseudomonas fluorescens* ACB as an oxidative biocatalyst in the synthesis of optically active sulfoxides, Tetrahedron Asymmetry. 17 (2006) 130–135.

[31] A. Doudouh, C. Woltermann, P.C. Gros, TMSCH(2)Li and TMSCH(2)Li-LiDMAE: efficient reagents for noncryogenic halogen-lithium exchange in bromopyridines., J. Org. Chem. 72 (2007) 4978–4980.

[32] J. Čubrilo, I. Hartenbach, F. Lissner, T. Schleid, M. Niemeyer, R.F. Winter, p-Cymene ruthenium thioether complexes, J. Organomet. Chem. 692 (2007) 1496–1504.

[33] H. Zhao, M.M. Kayser, Y. Wang, R. Palkovits, F. Schüth, Mesoporous silica modified with enantiopure sulfoxide as catalyst in allylation of aldehydes, Microporous Mesoporous Mater. 116 (2008) 196–203.

[34] C.O. Kinen, L.I. Rossi, R.H. de Rossi, The development of an environmentally benign sulfide oxidation procedure and its assessment by green chemistry metrics, Green Chem. 11 (2009) 223-228.

[35] A. Rioz-Martínez, G. de Gonzalo, D.E.T. Pazmiño, M.W. Fraaije, V. Gotor, Enzymatic Synthesis of Novel Chiral Sulfoxides Employing Baeyer-Villiger Monooxygenases, European J. Org. Chem. 2010 (2010) 6409–6416.

[36] F.R. Bisogno, A. Rioz-Martínez, C. Rodríguez, I. Lavandera, G. de Gonzalo, D.E. Torres Pazmiño, et al., Oxidoreductases Working Together: Concurrent Obtaining of Valuable Derivatives by Employing the PIKAT Method, ChemCatChem. 2 (2010) 946–949.

[37] D.R. Boyd, N.D. Sharma, N. Gunaratne, S.A. Haughey, M.A. Kennedy, J.F. Malone, et al., Dioxygenase-catalysed oxidation of monosubstituted thiophenes: sulfoxidation versus dihydrodiol formation., Org. Biomol. Chem. 1 (2003) 984–994.

[38] Integrated Spectral Database System of Organic Compounds, (n.d.).

[39] V. Pironti, S. Nicolis, E. Monzani, S. Colonna, L. Casella, Nitrite increases the enantioselectivity of sulfoxidation catalyzed by myoglobin derivatives in the presence of hydrogen peroxide, Tetrahedron. 60 (2004) 8153–8160.

[40] M.P.J. Van Deurzen, I.J. Remkes, F. Van Rantwijk, R.A. Sheldon, Chloroperoxidase catalyzed oxidations in t-butyl alcohol/water mixtures, in: J. Mol. Catal. A Chem., (1997) 329–337.

[41] J. Drabowicz, B. Bujnicki, M. Mikolajczyk, Stereochemistry of the conversion of sulfoxides into aminosulfonium salts, J. Org. Chem. 46 (1981) 2788–2790.

[42] G. de Gonzalo, G. Ottolina, F. Zambianchi, M.W. Fraaije, G. Carrea, Biocatalytic properties of Baeyer-Villiger monooxygenases in aqueous-organic media, J. Mol. Catal. B Enzym. 39 (2006) 91–97.

[43] H. Tsutsumi, M. Katagi, M. Nishikawa, H. Tsuchihashi, F. Kasuya, K. Igarashi, *In vitro* confirmation of selegiline N-oxidation by flavin-containing monooxygenase in rat microsome using LC-ESI MS., Biol. Pharm. Bull. 27 (2004) 1572–1575.

[44] E. Kashiyama, T. Yokoi, K. Itoh, S. Itoh, M. Odomi, T. Kamataki, Stereoselective *S*-oxidation of flosequinan sulfide by rat hepatic flavin-containing monooxygenase 1A1 expressed in yeast, Biochem. Pharmacol. 47 (1994) 1357–1363.

[45] P. Pitchen, E. Dunach, M.N. Deshmukh, H.B. Kagan, An efficient asymmetric oxidation of sulfides to sulfoxides, J. Am. Chem. Soc. 106 (1984) 8188–8193.

SUMMARY

Enzymology is a very large and interdisciplinary research field with strong interplay between topics such as protein chemistry, physiology, cell and molecular biology, synthetic and analytical chemistry, biochemistry, bioinformatics, structural biophysics, statistics and probably many more. The unique catalytic properties of an enzyme, like its specificity, selectivity, rate of chemical reaction and regulation, arise from its physiological role it plays in the respective living organism. Therefore, enzymes have been the subject of intense research in order to unveil their functions in metabolism and their shortcomings leading to different diseases. Having said that, enzymes are a very common target in medicine and pharmaceutical industry research. What is interesting to note, they have been used since ancient times for the production of wine, cheese and bread through the use of yeast. At the end of the 20th century enzymology entered a new era of protein engineering. This was due to a spectacular development in determining protein structures, gene manipulation and sequencing, as well as in computing power and software that can predict the impact of mutations introduced to an enzyme. This also opened the road to enzyme design for industrial purposes and nowadays many enzymes have been successfully applied and still a lot of research effort is spent on further development in this field. This thesis was intended to study the structure, activity and mechanism of several enzymes belonging to an oxidoreductases family. They all depend on a flavin cofactor which plays a pivotal role in the reactions they catalyse.

An overview of chemical reactions catalysed by cofactor containing enzymes is presented in **Chapter 2**. Cofactors have been acquired by enzymes in order to broaden their catalytical scope and are mostly exploited by oxidoreductases, the class of enzymes performing redox reactions. Selective enzymatic redox reactions are especially interesting for industrial processes, because they are not easily accessible by conventional chemical approaches. Furthermore, the capabilities of cofactor containing enzymes can be extended by the redesign of natural cofactors or by the design of new cofactor types. Such efforts will benefit from the well-developed methods of protein scaffold engineering to accept and tightly bind modified or new ligands. This field of enzyme engineering, which combines protein engineering and cofactor redesign, is highly valuable because it may generate totally new biocatalysts.

In this thesis, two different approaches of cofactor engineering have been explored. One of them was the covalent flavin binding to the protein

scaffold performed on two different model enzymes, putrescine oxidase (PuO) and 6-hydroxy-D-nicotine oxidase (6HDNO). For the first design, described in **Chapter 3**, PuO from *Rhodococcus erythropolis* was chosen, because it is a soluble homodimeric protein that binds flavin adenine dinucleotide (FAD) tightly, but in a non-covalent manner. The crystal structure elucidated by our collaborators in Pavia, Italy, revealed that PuO shows the structural features characteristic for other amine oxidases, in particular for the sequence-related human monoamine oxidases A and B. Surprisingly, this also includes the conservation of the flavin-binding site, although PuO binds the flavin non-covalently, in contrast to the monoamine oxidases. One of the mysteries regarding PuO is the competitive binding of ADP versus FAD in about half of wild-type monomers, which renders such monomer inactive. Based on the crystal structure, the residues that are responsible for this feature were identified. By replacing one of these residues, enzyme preparations were obtained in almost 100% FAD-bound form. Moreover, a triple mutant has been designed and prepared that formed a covalent 8α-S-cysteinyl-FAD linkage, similarly to monoamine oxidases.

Bicovalent flavoproteins are a recently discovered group of interesting enzymes, not only because of their unusual cofactor binding mode, but also because of their open active sites and bulky substrates they can accept. In **Chapter 4** we presented a sequence alignment of bicovalent flavoproteins, in which we have identified a conserved sequence region that is different from monocovalent flavoproteins. On this basis, the second model enzyme for covalent flavin-protein bond engineering was chosen: 6-hydroxy-D-nicotine oxidase (6HDNO). 6HDNO is a monocovalent flavoenzyme for which a crystal structure is available. In order to introduce a second cofactor-protein linkage, two amino acid replacements have been designed. The introduced cysteine forms a covalent bond with FAD as found in natural bicovalent flavoproteins, while the second mutation was found to be essential to facilitate formation of the cysteinyl linkage. The introduced histidine is supposed to stabilize a negative charge of the isoalloxazine ring during the process of covalent bond formation. The bicovalent 6HDNO mutant was found to be still active and showed a higher midpoint redox potential when compared with wild-type 6HDNO. This was expected, because the previous studies on bicovalent flavoenzymes have shown that they have extremely high redox potentials. It also confirmed that covalent flavinylation is typically a self-catalytic process. The successful introduction of an (additional) covalent flavin-protein bond in PuO and 6HDNO showed that

this protein engineering approach could be also considered in case of flavoproteins that loose flavin easily, rendering them inactive, like for example glucose oxidase. Furthermore, it could be utilized to develop more stable protein molecules, better suited for biocatalytic purposes.

The abovementioned approach of covalent flavin-protein bond engineering was based on the idea of linking the protein scaffold with the FAD naturally present in the enzyme. On the other hand, a lot of information can be obtained by using chemically modified flavins and reconstituting them into the flavin binding site in the studied flavoprotein. In **Chapter 5** the method for a specific deuteration of the C8 methyl group of FMN is presented. Furthermore the deuterated FMN can be converted enzymatically to riboflavin, which can be used in the cells as FAD cofactor precursor. The deuterated riboflavin used in combination with riboflavin auxotrophic strains would be an interesting approach to study effects of deuteration on the efficiency of covalent flavinylation. Furthermore, deuterated FMN can be converted by FAD synthetase into FAD, which would allow investigating the effects of C8 deuteration on the kinetics of covalent flavinylation directly with proteins purified or prepared as apo protein.

In addition to flavin-protein interaction engineering, also an investigation of the substrate specificity and catalytic properties of PuO is described. The elucidated structure of the enzyme (**Chapter 3**) explained the strong preference for aliphatic diamines by PuO. A glutamate residue present in the active site of PuO seems to be important for determining the distance between the flavin and the binding site for the positively charged substrate amino group, which is 4 carbon chain length as in putrescine. Previous studies have shown the importance of the glutamate for the activity of PuO by substitution to hydrophobic residues. In **Chapter 6**, we report on a PuO mutant, in which the charge is retained but positioned more distant from the flavin cofactor (glutamate-aspartate replacement). The enzyme still shows significant activity with putrescine and cadaverine, but it loses the ability to discriminate between these two diamines and for both substrates similar catalytic efficiencies are observed. The sequence comparison between (putative) putrescine oxidases and other more distant homologs suggests that the glutamate is a conserved residue that can be used to annotate putrescine oxidase sequences.

So far, the kinetic mechanism of PuO has not been established. In C**hapter 7** we present a detailed kinetic analysis of PuO using both single- and double-mixing stopped-flow spectrophotometry and putrescine as a substrate. The reductive-

half reaction is fast and irreversible. A direct hydride transfer from putrescine to the FAD was proposed for PuO, because no radical intermediates were observed in the reductive half-reaction. Ligand binding was found to influence the rate of flavin reoxidation; when the product was bound to the enzyme the rate was higher than with free enzyme species and similar results were obtained with product-mimicking ligands. This indicates that a ternary complex is formed during catalysis. The bound product might deliver a proper electrostatic environment for flavin reoxidation or it may increase accessibility of the flavin for dioxygen. The obtained kinetic data were used together with steady-state rate equations derived for ping-pong, ternary complex and bifurcated mechanisms to explore which mechanism is operative. The integrated analysis revealed that at atmospheric conditions PuO operates *via* a bifurcated mechanism due to comparable rates of product release from the reduced enzyme and reoxidation of the reduced enzyme-product complex. However, at high oxygen concentrations PuO will mostly follow the ternary complex mechanism, while the ping-pong mechanism becomes more dominant at decreased O_2 concentrations. This might be an adaptation of *R. erythropolis* for living in environments with low oxygen concentration.

Because PuO shows narrow substrate specificity for aliphatic diamines and polyamines, it could be used in diagnostic applications in the detection of cancer and spoiled food. The high level of expression and the resolved structure of PuO are excellent starting points for substrate scope engineering, especially since the enzyme is robust and can accommodate several mutations at the same time.

In addition to flavoprotein oxidases, we also wanted to gain insight into a biocatalytic scope of a bacterial flavin-containing monooxygenase (mFMO) (**Chapter 8**). The enzyme was fused to phosphite dehydrogenase (PTDH-mFMO) and could be expressed in high amounts in *Escherichia coli*. The bifunctional biocatalyst was able to oxidize indole and indole derivatives into a variety of colourful indigo compounds. Furthermore, the monooxygenase performed the sulfoxidation of a wide range of prochiral sulfides, showing moderate to good enantioselectivities in forming chiral sulfoxides. The fused bifunctional dehydrogenase-mFMO system represents an interesting oxidative biocatalyst that could be used for synthetic purposes, like indigoid pigments production.

SAMENVATTING

Enzymologie is een groot en interdisciplinair onderzoeksgebied met een sterke wisselwerking tussen onderwerpen zoals eiwitchemie, fysiologie, cel- en moleculaire biologie, synthetische en analytische chemie, biochemie, bio-informatica, structurele biofysica, en statistiek. De unieke katalytische eigenschappen van een enzym, zoals zijn specificiteit, selectiviteit, snelheid van de chemische reactie en regulatie, vloeien voort uit de fysiologische rol die het speelt in het levende organisme. Enzymen zijn daarom onderwerp van intensief onderzoek om hun functies te onthullen in de stofwisseling en de tekortkomingen die tot verschillende ziekten kunnen leiden. Daardoor zijn zij een veel voorkomend doelwit in de geneeskunde en de farmacie. Het is interessant om op te merken dat enzymen al sinds de oudheid gebruikt zijn voor de productie van wijn, kaas en brood door het gebruik van gist. Aan het eind van de 20e eeuw is een nieuw tijdperk aangebroken voor enzymologie. Dit werd mogelijk gemaakt door spectaculaire ontwikkelingen in het bepalen van eiwitstructuren, genmanipulatie en sequencing. Ook de toenemende computerkracht en nieuwe software die de gevolgen van mutaties, geïntroduceerd in een enzym, kunnen voorspellen hebben hieraan bijgedragen. Dit opende de weg naar het ontwerpen van enzymen voor industriële doeleinden. Tegenwoordig worden veel enzymen succesvol in de industrie toegepast en nog steeds wordt veel inspanning geleverd om dit gebied verder te ontwikkelen. Dit proefschrift is bedoeld om de structuur, de activiteit en het mechanisme te bestuderen van een aantal oxidatieve enzymen. Ze zijn allemaal afhankelijk van een flavine cofactor, die een centrale rol speelt in de reacties die ze katalyseren.

Een overzicht van chemische reacties die gekatalyseerd worden door cofactor bevattende enzymen wordt beschreven in **hoofdstuk 2**. Cofactoren worden gebruikt door enzymen om hun katalytische mogelijkheden te verbreden en worden meestal geëxploiteerd door oxidoreductases, de klasse van enzymen die redoxreacties uitvoert. Selectieve enzymatische redoxreacties zijn vooral interessant voor industriële processen, omdat het niet gemakkelijk is om deze selectiviteit te verkrijgen met gebruikelijke chemische benaderingen. De mogelijkheden van de cofactor die enzymen bevatten kunnen worden uitgebreid door het aanpassen van natuurlijke cofactoren of door het ontwerp van nieuwe cofactor types. Dergelijke inspanningen zullen profiteren van de goed ontwikkelde methoden om *protein scaffold* te ontwerpen die gewijzigde of

nieuwe liganden accepteren en sterk binden. Dit veld van enzymontwerp, dat *protein engineering* en cofactor herontwerp combineert, is zeer waardevol. Het is namelijk mogelijk om op deze manier totaal nieuwe biokatalysatoren te genereren. In dit proefschrift zijn twee verschillende benaderingen onderzocht waarop de cofactor aangepast kan worden. Een daarvan was de covalente binding van flavine aan de *protein scaffold*, uitgevoerd op twee verschillende type enzymen, namelijk putrescine oxidase (PuO) en 6-hydroxy-D-nicotine oxidase (6HDNO). Voor het eerste ontwerp, beschreven in **hoofdstuk 3**, is gekozen voor PuO afkomstig uit *Rhodococcus erythropolis*, omdat het een oplosbaar homodimeer eiwit is dat flavine adenine dinucleotide (FAD) sterk, maar op niet-covalente wijze bindt. De kristalstructuur is opgehelderd door onze samenwerkingspartners in Pavia, Italië, en het bleek dat PuO de structuurelementen bevat die kenmerkend zijn voor andere amine oxidases, met name de in sequentie verwante humane monoamine oxidases A en B. Verrassenderwijs is ook de flavine-bindingsplaats geconserveerd, hoewel PuO de flavine niet-covalent bindt, in tegenstelling tot de monoamine oxidases. Een van de mysteries betreffende PuO is de competitieve binding van ADP versus FAD in ongeveer de helft van de wild-type monomeren, die de desbetreffende monomeren inactief maakt. Gebaseerd op de kristalstructuur, werden de residuen die verantwoordelijk zijn voor deze functie geïdentificeerd. Door het vervangen van een van deze residuen, werden enzympreparaten verkregen in bijna 100% FAD-gebonden vorm. Daarnaast is een drievoudige mutant ontworpen en geprepareerd die een covalente 8α-S-cysteïnyl-FAD koppeling bevat, vergelijkbaar met de covalente binding van FAD in de humane monoamine oxidases.

Bicovalente flavoproteïnen zijn een recent ontdekte groep interessante enzymen, niet alleen vanwege hun ongewone cofactor bindingsmodus, maar ook omdat ze vanwege hun open actieve centra zeer grote substraten accepteren. In **hoofdstuk 4** presenteren we een *sequence alignment* van bicovalente flavoproteinen, waarin we een geconserveerde sequentie regio hebben geïdentificeerd die verschilt van monocovalente flavoproteïnen. Op basis hiervan is het tweede modelenzym voor het ontwerpen van covalente flavine-eiwit bindingen gekozen: 6-hydroxy-D-nicotine-oxidase (6HDNO). 6HDNO is een monocovalent flavo-enzym waarvan een kristalstructuur beschikbaar is. Om een tweede cofactor-eiwit binding te introduceren, zijn twee aminozuren vervangen. De geïntroduceerde cysteïne vormt nu inderdaad een covalente binding met FAD, zoals in natuurlijke bicovalente flavoproteïnen, terwijl de tweede mutatie essentieel bleek te zijn om de vorming

van de cysteïnyl-flavine koppeling te vergemakkelijken. De geïntroduceerde histidine wordt verondersteld een negatieve lading van de isoalloxazine ring te stabiliseren tijdens het vormen van de covalente binding. De bicovalente 6HDNO mutant bleek nog steeds actief te zijn en vertoonde een hogere redoxpotentiaal in vergelijking met wild-type 6HDNO. Dit werd ook verwacht, omdat eerdere onderzoeken naar bicovalente flavo-enzymen hebben aangetoond dat zij een zeer hoge redoxpotentiaal bezitten. Het bevestigde ook dat covalente flavinylering een zelf-katalytisch proces is. De succesvolle introductie van een (tweede) covalente flavine-eiwit binding in PuO en 6HDNO toonde aan dat deze *protein engineering* aanpak ook kan worden overwogen in het geval van flavoproteinen die gemakkelijk de flavine cofactor verliezen en daardoor inactief worden, zoals bijvoorbeeld glucose oxidase. Bovendien kan het worden gebruikt om stabielere eiwitmoleculen te ontwikkelen, die beter geschikt zijn voor biokatalytische doeleinden.

De bovenstaande benadering om covalente flavine-eiwit bindingen te ontwerpen is gebaseerd op het koppelen van de specifieke *protein scaffold* met de FAD cofactor, dat natuurlijk aanwezig is in het enzym. Anderzijds kan veel informatie worden verkregen door chemisch gemodificeerde flavines te reconstitueren in de flavine bindingsplaats van de bestudeerde flavine-proteïne. In **hoofdstuk 5** is de methode gepresenteerd waarmee de C8 methylgroep van FMN specifiek gedeutereerd kan worden. Verder kan de gedeutereerde FMN enzymatisch worden omgezet in riboflavine, dat in cellen gebruikt kan worden als FAD cofactor precursor. De gedeutereerde riboflavine, in combinatie met riboflavine auxotrofe stammen, zou een interessante benadering zijn om de effecten van deuteringsgraad op de efficiëntie van covalente flavinylering te bestuderen. Bovendien kan gedeutereerd FMN worden omgezet door FAD synthetase in FAD. Dit maakt het mogelijk om de effecten van de C8 deuteringsgraad op de kinetiek van covalente flavinylation rechtstreeks te bestuderen met gezuiverde enzymen waar het FAD uit is verwijderd of eiwitten die al in de FAD-loze vorm gezuiverd worden.

Naast flavine-eiwitinteractie ontwerp is ook een onderzoek naar de substraatspecificiteit en katalytische eigenschappen van PuO beschreven. De opgehelderde structuur van het enzym (**hoofdstuk 3**) verklaart de sterke voorkeur van PuO voor alifatische diamines. Een glutamaat residu in het actieve centrum van PuO lijkt belangrijk te zijn voor het bepalen van de afstand tussen de flavine en de bindingsplaats voor de positief geladen aminogroep van het substraat, die vier koolstofketenlengtes is, zoals in putrescine. Eerdere studies hebben het

belang van dit glutamaat residu aangetoond voor de activiteit van PuO door substitutie met hydrofobe residuen. In **hoofdstuk 6** rapporteren we over een PuO mutant, waarin de lading wordt behouden, maar verder van de flavine cofactor wordt gepositioneerd. Dit wordt gedaan door de glutamaat door een aspartaat te vervangen. Het enzym vertoont nog steeds significante activiteit met putrescine en cadaverine, maar heeft het vermogen om onderscheid te maken tussen deze twee diamines verloren en vertoont voor beide substraten soortgelijke katalytische efficiëntie. De vergelijking van de sequenties van (vermeende) putrescine oxidases en andere, minder verwante homologen suggereert dat de glutamaat een geconserveerd residu is, dat kan worden gebruikt om putrescine oxidase sequenties te annoteren.

Tot dusver is het kinetische mechanisme van PuO niet onderzocht. In **hoofdstuk 7** presenteren we een gedetailleerde kinetische analyse van PuO met behulp van zowel single als double-mixing stopped flow spectrofotometrie en met putrescine als substraat. De reductieve half-reactie is snel en niet reversibel. Een directe overdracht van een hydride van putrescine naar FAD is voorgesteld voor PuO, omdat er geen radicaal-intermediairen werden waargenomen in de reductieve half-reactie. Het binden van een ligand beïnvloedt de snelheid van flavine reoxidatie: wanneer het product was gebonden aan het enzym, was de snelheid hoger dan bij vrij enzym en vergelijkbare resultaten werden verkregen met liganden die lijken op het product. Dit geeft aan dat een ternair complex wordt gevormd tijdens de katalyse. Het gebonden product kan een goede elektrostatische omgeving leveren voor flavine reoxidatie of het kan de toegankelijkheid van de flavine voor zuurstof verhogen. De verkregen kinetische gegevens werden gebruikt in combinatie met steady-state vergelijkingen, afgeleid voor ping-pong, ternair complex en een mix van deze mechanismen, om te onderzoeken welk mechanisme wordt gevolgd. Uit de geïntegreerde analyse bleek dat bij atmosferische omstandigheden PuO werkt via beide mechanismen (ping-pong en ternair complex mechanismen) omdat vergelijkbare snelheden werden gevonden voor product dissociatie van het gereduceerde enzym en reoxidatie van het gereduceerde enzym-product complex. Echter, bij hoge zuurstofconcentraties zal PuO vooral een ternair complex mechanisme volgen, terwijl het ping-pong mechanisme meer dominant wordt bij verlaagde O_2 concentraties. Dit kan een aanpassing zijn van *R. erythropolis* voor het leven in omgevingen met een lage zuurstofconcentratie. Omdat PuO een strakke substraatspecificiteit heeft voor alifatische diamines en

polyamines, kan het worden gebruikt in diagnostische toepassingen bij kanker of de detectie van bedorven voedsel. Het hoge niveau van expressie en de opgehelderde structuur van PuO zijn uitstekende uitgangspunten voor substraat scope engineering, vooral omdat het enzym robuust is en meerdere mutaties op hetzelfde moment kan accommoderen.

Naast flavo-eiwit oxidases, wilden we ook inzicht krijgen in de biokatalytische reikwijdte van een bacteriële flavine-bevattende monooxygenase (mFMO) (**hoofdstuk 8**). Het enzym werd gefuseerd aan fosfiet dehydrogenase (PTDH-mFMO) en kon in grote hoeveelheden in *Escherichia coli* tot expressie worden gebracht. De bifunctionele biokatalysator kon indool en indoolderivaten oxideren ~~in~~ naar een verscheidenheid aan kleurrijke indigo verbindingen. Bovendien kon de monooxygenase de sulfoxidatie van diverse prochirale sulfiden uitvoeren, met matige tot goede enantioselectiviteit bij het vormen van chirale sulfoxiden. Het gefuseerde bifunctionele dehydrogenase-mFMO systeem blijkt een interessante oxidatieve biokatalysator die kan worden gebruikt voor synthetische doeleinden, zoals de productie van indigoïde pigmenten.

LIST OF PUBLICATIONS

M. M. Kopacz, D. P. H. M. Heuts, M. W. Fraaije, Kinetic Mechanism of Putrescine Oxidase from Rhodococcus erythropolis, Submitted to FEBS Journal

M. M. Kopacz, M. W. Fraaije, Turning a Monocovalent Flavoprotein into a Bicovalent Flavoprotein by Structure-Inspired Mutagenesis, Accepted in Bioorganic & Medicinal Chemistry

M. M. Kopacz, F. Hollman, M. W. Fraaije, Enzyme Engineering by Cofactor Redesign, Protein Engineering Handbook (2013), 3, 163-192

M. M. Kopacz, I. Kholodowa, S. Rovida, A. Mattevi, M. W. Fraaije, Functional Role of the Active Site Glu324 of Putrescine Oxidase, Flavins and flavoproteins 2011 (2013), 163-168

M. M. Kopacz, S. Rovida, E. van Duijn, M. W. Fraaije, A. Mattevi, Structure-Based Redesign Of Cofactor Binding In Putrescine Oxidase, Biochemistry (2011), 50(19), 4209-17

A. Rioz-Martínez, M. Kopacz, G. de Gonzalo, D. E. Torres Pazmiño, V. Gotor, M. W. Fraaije, Exploring the Biocatalytic Scope of a Bacterial Flavin-Containing Monooxygenase, Organic & Biomolecular Chemistry (2011), 9, 1337-41

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